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Effects of rumen bacterial lipases on ruminal lipid metabolism

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This thesis is dedicated to the memory of my Grandma, Mavis Williams, and of my horse, Follie, who both taught me dedication, confidence and patience – all of which has enabled me to succeed thus far.

Declaration

This work has not previously been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

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Summary

With the world population set to increase to 9.6 billion in 2050 and increasing fatty food related diseases, it is paramount that a secure and nutritious food supply is available. Despite the ruminant diet being rich in polyunsaturated fats (PUFA), ruminant products are high in saturated fat (SFA). This is due to lipolysis and subsequent biohydrogenation of PUFA in the rumen. Bacteria may biohydrogenate PUFA due to the toxicity of its double bonds upon incorporation into the bacterial membrane, so it is feasible that biohydrogenation could be inhibited using high concentrations of PUFA.

To test this hypothesis, a batch culture experiment was undertaken using 50% buffered rumen fluid and linoleic (LA) or linolenic acid (LNA) at 50µM, 250µM, 500µM, 750µM or 1mM. Lipids were extracted and analysed using FAME and gas chromatography. Total lipid profiles showed a decrease in 18:0 over 24 hours (250µM LNA) and at 4 hours reduced by 11.5% (250µM LA) and 16.7% (250µM LNA). Overall, data indicates that it is possible to inhibit biohydrogenation using PUFA to an extent, implying that lipolysis may be a suitable control point.

Another batch experiment was performed using increasing concentrations of the A1 phospholipase from *Thermomyceslanuginosus*(75 µM, 100 µM, 125 µM and 150 µM) with 50% buffered rumen fluid and phospholipid extract. Lipids were extracted and analysed using TLC (thin layer chromatography) and FAME (fatty acid methyl esters). There were slight decreases in C18 content (8.9%) suggesting partial inhibition of biohydrogenation. Decreases in LNA (up to 25%), LA and C16 with in the polar lipid fraction following addition of 100, 125 and 150 µM concentrations of phospholipase A1, and particularly after 24h of incubation suggesting lipolysis was enhanced. In terms of biohydrogenation analysis of the fatty acids within the free fatty acid fraction showed that after addition of all concentrations of A1 phospholipase there were decreases in C18 content (8.9%) suggesting partial inhibition of biohydrogenation. Inhibition was achieved at a specific point resulting in an accumulation of the intermediate 18:1, *trans*-11 (up to 25% of total lipids) which is beneficial as it can be converted by Δ9-transferase in the bovine mammary gland to CLA, which is a human health beneficial fatty acid.

Whilst lipolysis enhancement shows potential, it is clear even higher concentrations of phospholipase are necessary to successfully inhibit biohydrogenation; the practicalities of which are questionable. Further research into lipolysis as a control point for biohydrogenation is also necessary, as well as into lipolytic bacteria and their lipases.

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List of abbreviations

A_{410} Absorbance measured at 410 nm

A Adenosine

BLAST Basic Local Alignment and Search Tool

BOC Branched and Odd Chain Fatty Acid

°C Degrees Celsius

C Cytosine

cDNA Complementary DNA

CHD Coronary Heart Disease

CLA Conjugated Linoleic Acid

CVD Cardiovascular Disease

DM Dry Matter

DNA Deoxyribose Nucleic Acid

dNTPDeoxynucleotide

FA Fatty Acid

g Grams

GHG Greenhouse Gas Emissions

h Hours

Ig Immunoglobulin

IPTG Isopropyl β-D-1-thiogalactopyranoside

KbKilobase

kDa Kilo Daltons

Kg Kilograms

L Litres

LA Linoleic Acid

LCPUFA Long Chain Polyunsaturated Fatty Acids

LiCl Lithium Chloride

LNA Linolenic Acid

LBLysogeny Broth

M Molar

MES 2-(N-morpholino)ethanesulfonic acid

mg Milligrams

mL millilitre

mMMillimolar

N_2O Nitrous Oxide

ngNanograms

NH_3Ammonia

nm Nanometre

OD Optical Density

PCR Polymerase Chain Reaction

PNP ρ-nitrophenol

PRG Perennial Rye Grass

PUFA Polyunsaturated Fatty Acid

rDNA Recombinant Deoxyribose Nucleic Acid

RNA Ribonucleic Acid

rpm Revolutions per Minute

SDS-PAGE Sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis

SFA Saturated Fatty Acid

TEMED N', N', N', N', Tetramethylethylenediamine

TRFLP Terminal Restriction Fragment Length Polymorphism

U Units

UV Ultraviolet

V Volts

VFA Volatile Fatty Acid

v/v Volume to volume ratio

µg Micrograms

µM/µmol Micro Molar

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1. Introduction

1.1: Agricultural Challenges

Meat and milk provide many of our essential vitamins and minerals and form a major proportion of the modern human diet. The predicted increase in farm animal production due to ourrapidly growing population means we need to raise more animals, but have limited land on which to do so. Thus the agricultural sector faces major challenges for increasing animal production in the next 40 years.By 2050, global farm animal production is predicted to double from the current 56 billion livestock raised and slaughtered every year in order to feed the accompanying rise in the world population (Ilea, 2000). Livestock graze 26% of the terrestrial surface of the earth and 33% of cropland is used in their feed production, contributing significantly to deforestation and degradation due to overgrazing, compaction and erosion (FAO, 2012; UN, 2013[b]). As well as an impact on the land, livestock contributes greatly to a loss of biodiversity, mainly due to out-competing wildlife for resources, damage to the land, over grazing, deforestation and general pollution (McLaughlin and Mineau, 1995; Reidsma*et al.*, 2006).

Methane released from ruminants also account for a large part of emissions, – 38% of global methane emissions (Department of Energy and Climate Change, 2014). Continuous research is conducted into reducing methane emissions from ruminants; but science struggles to keep up with the rapidly growing sector, pressured by our growing population.

Alongside these challenges is the need to ensure that ruminant products are safe and nutritious. Meat and milk are often said to be detrimental to human health due to their levels of saturated fatty acids. With an increase in consumption of meat and milk, it is also essential that novel strategies to reduce the saturated fatty acid content are developed.

1.1.1: World population growth

The world population is projected to increase from 7 billion in 2013 to 9.6 billion by 2050, an expansion of almost 30% (UN, 2013[a]). World hunger is already a major challenge for the modern world, which coupled with extreme weather patterns and the emergence of new diseases, makes securing a stable and nutritious food supply for the future a key challenge. Much of the predicted population growth is set to occur in less developed regions (Africa, Asia and India), with the sub-Saharan African population predicted to increase from 770 million to 1.7 billion by 2050. Developing countries generally possess a poorer supply of

food and water at present, and thus will need to adapt radically to accommodate this change in demographic (Fig 1.1) (UN, 2004).

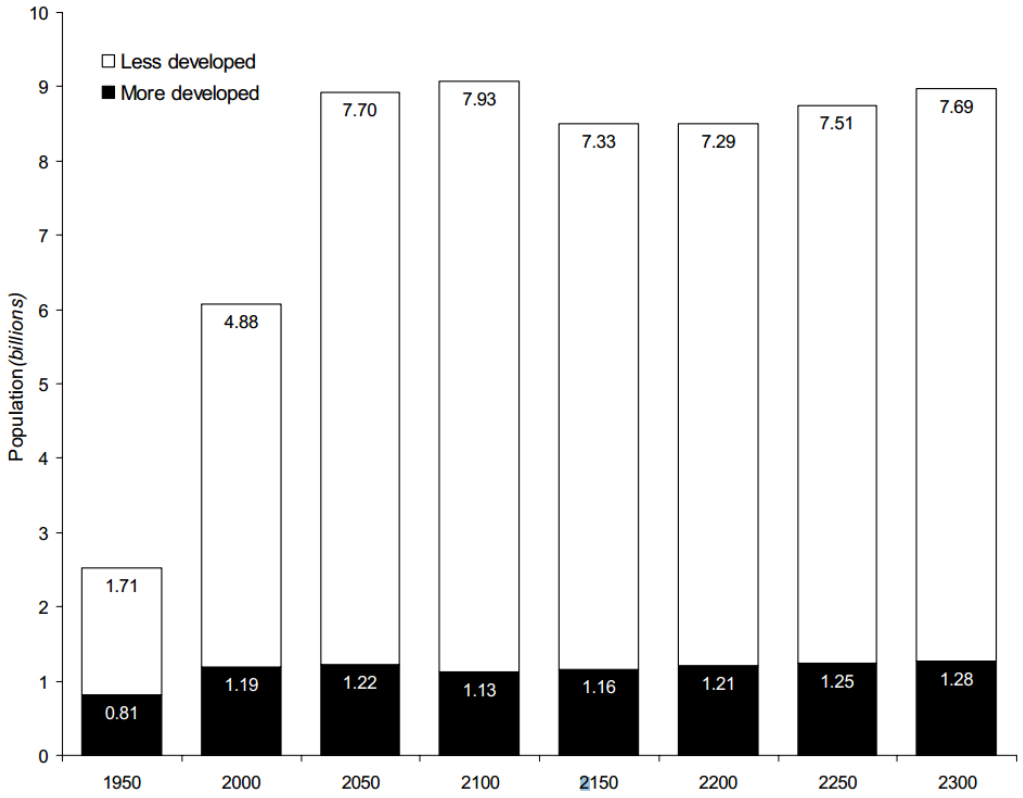


Fig 1.1: Total population in more developed and less developed regions, estimates and medium scenario: 1950-2300 (UN, 2004).

In recent years, the availability of meat has increased and its premium decreased, which has led to an increase in consumption and therefore demand. In developed countries annual per capita consumption of meat has doubled from 14 kilograms (kg) in 1980 to 28kg in 2002, and global meat supply tripled during this time period (Steinfeld*et al.*, 2006). This transition has been aptly named 'The livestock revolution' by Delgado *et al.* (1999) and is predicted to continue into 2020. Availability of meat can partially explain our increase in consumption, but as general income also increases populations are able to afford more nutritious and higher quality food items.

1.1.2: Greenhouse gas emissions

The UN states, in its 'Livestock's Long Shadow' review (2006) that the environmental impact of livestock must be cut by half, simply to avoid increasing beyond the present level of damage, with further changes required to begin reversal of these detrimental effects.

Global warming is one of the most adverse events faced by humanity in the modern day, and it is well documented that livestock alone contribute a substantial amount of greenhouse gases(GHG), approximately 18% (Steinfeld*et al.*, 2006). Our primary concern lies with carbon dioxide (CO_2) due to the high concentrations and vast quantities we produce, however it possesses the least global warming potential when taking into account pre-industrial and current levels (Steinfeld*et al.,* 2006). Around 20% of GHGs emitted by livestock is composed of methane (CH_4), considered perhaps the most important GHG. Methane remains in the atmosphere for 9-15 years and is 21 times more effective than CO_2 at trapping heat (Steinfeld*et al.*, 2006).

Typically, livestock ruminants lose around 6% of their ingested energy as CH_4, with 95.5% of that coming from the rumen, which equates to a release of up to 600 litres (L) per animal per day (Johnson and Johnson, 1995; Wright *et al.,* 2004). Nitrous Oxide (N_2O) also poses a great deal of global warming potential and is even more effective at trapping heat than CH_4 with an even longer atmospheric lifetime, although fluctuations in tropospheric concentrations are not as dramatic (Steinfeld*et al.*, 2006). In terms of livestock, N_2O is released in urine and dung (due to the high nitrogen concentration of feed), which is accountable for up to 9% of total N_2O emissions (Oenema*et al.*, 2007). Recent research focussed on CH_4 mitigation has elucidated a variety of options, including anti-methanogen vaccinations and the use of feed additives (such as ionophores and tannin/saponin extracts). The latter of which has seen the most success due to the plant's natural quality and ease of administration (Martin *et al.*, 2009). Livestock can also be held responsible for almost two thirds of ammonia (NH_3) emissions, found in fertilisers and livestock excreta. Ammoniacontributes significantly to acid rain and acidification in ecosystems and can also cause a drop in pH (Steinfeld*et al.*, 2006).

1.1.3: Ruminant products and human health

Generally, as a country becomes more economically developed its health status also improves. However, as developed countries become more industrialised, we are seeing a shift in health problems – An Epidemiological transition (Yusuf *et al.*, 2001). This transition describes a shift from infectious diseases and nutritional deficiencies to degenerative diseases, as well as auto-immune conditions and hypersensitivity reactions (Yusuf *et al.*, 2001).

The shift towards chronic illnesses on the other hand, can be partially attributed to the development of agriculture and animal husbandry, which has facilitated a shift in the lifestyle and diet of western society to consuming more meat and milk, along with carbohydrates, salt

and sugar. Red meat itself is nutrient-rich, providing many vitamins and minerals, in particular Iron, zinc and B vitamins. In a correct, balanced diet red meat is highly nutritious and beneficial. However increased consumption of red meat consisting mostly of processed foods and fatty cuts, which contain more SFAs, salt and sugar, and less of the important vitamins and minerals, is detrimental. In developed countries, such as the UK, one person consumes on average 224 g of meat per day, a stark contrast to the recommended 90g; of which no more than 50g should be red meat (McMichael *et al.*, 2007).

This shift to an unbalanced diet, with a bias towards high fat and sugar food has caused an increase in chronic diseases and conditions, such as cardiovascular diseases, cancers, type 2 diabetes, hypertension and hypercholesterolaemia. Of particular note are cardiovascular diseases, with an estimated 2.7 million people currently living in the UK with coronary heart disease (CHD) (NHS, 2012). According to WHO 2013, 7.3 million people worldwide have died from coronary heart disease this year, which accounts for just over 15% of all deaths worldwide. CHD is responsible for around 82,000 deaths per annum in the UK; symptoms of CHD vary greatly, with some patients experiencing angina, breathlessness and palpitations, but with others displaying no symptoms until suffering a sudden heart attack (NHS, 2012). CHD is characterised by atherosclerosis, in which the coronary arteries become blocked and hardened by plaques, subsequently reducing blood flow to internal organs and, if a plaque ruptures, clot formation leading to a heart attack or a stroke.

1.2: Ruminants and the rumen microbiota

Ruminants are aptly named after their complex digestive system, and can be defined as a member of the order *Artiodactyla* which possesses a stomach which is divided into the rumen, reticulum, omasum and abomasum (Fig 1.2) (Hackmann and Spain, 2010). In the rumen and reticulum food is mixed with saliva and separates into the solid and liquid phase, of which the solids form a food bolus. This bolus moves between the oral cavity, reticulum and rumen and is broken down both mechanically through chewing and by the microbial population of the rumen. The digesta is then moved into the omasum where most absorption of water and inorganic elements occurs. The remaining bolus then moves to the abomasum (the ‘true stomach’) which works in a similar way to a monogastric stomach using peristaltic movements along with proteases and hydrochloric acid (Fig 1.2).

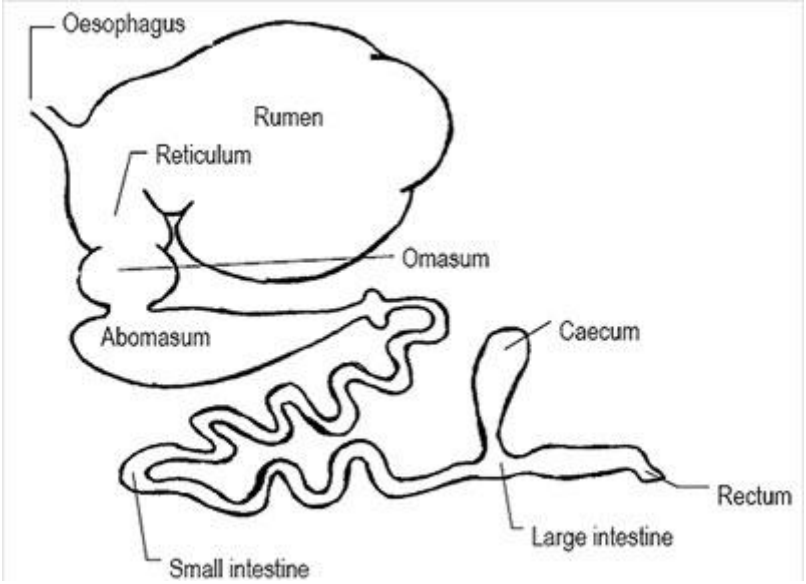


Fig 1.2: Bovine digestive tract(Moran, 2005).

The structure and function of the ruminant digestive system has continued to be a point of dynamic and interesting research over the years, and as such, it is a well-studied area. Of particular interest, is the rumen, the first chamber of the digestive tract through which food particles, saliva and drinking water flow to meet with a diverse microbial community (Hungate, 1997). The rumen evolved to allow better access to energy, locked in high fibre feeds such as grasses, low growing plants and shrubs and linseed or grain in domesticated ruminants (Van Soest, 1994). The term ruminant encapsulates many species, including goats, buffalo, reindeer, yaks and most numerous, cattle and sheep, the vast majority of which are domesticated for human use (Van Soest, 1994). Therefore, the bovine rumen is the subject of interest, in particular those raised for the purpose of meat and milk.

Thought to have been domesticated around 6,000 BC for draught work, all modern day domestic cattle evolved from one early, common ancestor – *Bos primigenius* (commonly named the Auroch), extinct as of 1927 in Europe (Bollongino*et al.*, 2006). From *B. primigenius*, three subspecies emerged in accordance with geographical location: Eurasian Aurochs, Indian Aurochs and North African Aurochs (Loftus *et al.*, 1994). Of interest here, is the Eurasian subspecies, or Taurine cattle which here in Europe have diverged into two distinct species: *Bos taurus* and *Bos indicus*. *B. taurus* account for the majority of domestic cattle in modern day, and are used widely for beef and dairy, *Bos indicus* on the other hand can be commonly identified as the humped Zebu cattle, most abundant in areas of Africa after their import. Within *B. taurus*there are numerous breeds, with beef cattle all adhering to a similar profile: compact, well fleshed and blocky in conformation with short sturdy legs and a very flat top line.

Of the four compartments in the digestive tract, the rumen carries out the majority of fermentation; it provides the perfect environment for an anaerobic, symbiotic microbiome (Dehority, 2003). Because of this highly evolved digestive system, little chewing is required during ingestion and the forage is combined with large volumes of saliva to form a bolus. The bolus then passes through the oesophagus to the reticulum before entering the rumen. Once food particles have entered the rumen they face one of two fates: fermentative digestion (up to 85% of material) or passage through the system. Once in the rumen, synchronised contractions of the rumen-reticulum facilitate the mixing of food and rumen fluid, leading to regurgitation (allowing ruminants to 'chew the cud'), the release of gas and eventually movement of digesta past the rumen, into the omasum and abomasum (the 'true' stomach) (Dehority, 2003).

The reticulum's epithelial lining is raised into folds to form a honeycomb structure, responsible for capturing indigestible material and foreign bodies. In contrast, the rumen is lined with papillae for greater adsorption (Fig 1.3) (Dehority, 2003).The rumen environment is constant, averaging at 38-40°C with a pH of 6-6.7, although the pH can be quite easily manipulated via the diet (Hungate, 1997).

Fig 1.3: A: interior surface of the rumen showing papillae. B: Reticular epithelium showing honeycomb structure and papillae. C: Interior showing longitudinal folds (Bowen, 2003).

The rumen contains, as previously mentioned, a dynamic, yet balanced microbial community, consisting of protozoa, archaea, anaerobic fungi and most predominantly, bacteria. The population equates to between 10^{10} and 10^{12} microbes per mL, consisting of approximately: 10^{10}- 10^{11} bacterial cells/mL, 10^{4}-10^{6} protozoa/mL, 10^{3}-10^{5} zoospores/mL and 10^{8}-10^{9} bacteriophages/mL (Kamra, 2005). The majority of the microbes in the rumen are as yet uncultured; Edwards *et al.*(2004) showed that 89% of sequences from a bacterial rDNA library were from organisms not yet cultured, indicating that only 11% of ruminal bacteria have been successfully grown *in vitro*. Thus, it is clear that the microbial population of the

rumen is much larger and more complex than we currently perceive. The rumen microbiota differs slightly from animal to animal, almost like a fingerprint, the species and populations of microorganisms very between ruminants.

Changes in the rumen microbiome can occur by altering the diet, for example using feed additives, protein supplements, fish oil and plant extracts. A study by Huws *et al.* (2010) found that feeding steers a combination of fish oil and red clover caused changes in several liquid associated and solid associated bacteria, including changes in the *Butyrivibrioproteoclasticus*group, *Anaerovibriolipolytica* and *Ruminococcus* spp. More recently, changes in the rumen microbiome have been investigated upon the addition of echium and flax oil (Huws *et al.*, 2014). Next Generation sequencing in this study, allowed a larger depth of sequencing and revealed an abundance of the *Butyrivibrio*, *Howardella*, *Oribacterium*, *Pseudobutyrivibrio* and *Roseburia*post-flax feeding. Post-echium oil feeding showed increases in the *Succinovibrio*and*Roseburia* genera, calling into question therole of these bacteria in biohydrogenation.

Rates of digestion also vary greatly, depending on several different factors: finely ground feeds will increase the digestive rate, as will lactation and pregnancy. Decreasing temperature, and including a protein supplement or ionophores can help decrease passage of material, allowing more time for fermentation (Fox *et al.*, 2004; Serjsen*et al.*, 2006). The true challenge arises when trying to manipulate specific species in the rumen, which could potentially cause severe disruption to the population and it's finely tuned, symbiotic nature.

1.2.1: The rumen bacteria

Bacterial populations of the rumen far exceed those of protozoa and fungi. The axenic culture of rumen bacteria was developed by Hungate in the 1940s upon development of a strictly anaerobic technique (The Hungate culture technique) using medium containing reducing agents and sterile rumen fluid (Hungate, 1947). This technique is arguably responsible for the discovery and identification of most rumen microbes to date, although attempts using further enhanced types of media have resulted in successful growth of rumen bacteria (Janssen *et al.*, 2008). Using these techniques, microbes can be cultured and subsequently identified using simple staining techniques and microscopy, focussing on morphology and pigmentation, as well as PCR and sequencing methods.

Tajima *et al.*(1999) constructed an extensive 16S rDNA clone library using rumen fluid, and most sequences were associated with the following phyla: low G+C Gram-positive bacteria (52.4%), *Cytophaga-Flexibacter-Bacteroides* (38.1%), *Proteobacteria* (4.7%)

and *Spirochaetes* (2.4%). Similar results were published by Whitford *et al* (1998) who found 55% of 16SrDNA was similar to low G+C gram positive bacteria that were related to *Clostridia* and 30% constituted *Prevotella-Bacteriocedes*group. Furthermore, Edwards *et al* (2004) found 54% of their bacterial 16S rDNA library consisted of low G+C gram positive bacteria, 40% from the *Cytophaga-Flexibacter-Bacteroides*phyla. Most rumen bacteria are cocci or rods (although cresentic, spiral, spirochete and irregular shapes are also present) occurring singly, in chains or in clusters, usually in the range of 0.4-1.0 micrometres (μm) in diameter and 1-3μm in length (Hungate, 1966; Kamra, 2005). Motility varies greatly, from sessile bacteria to those with petritrichous flagella; the presence of a capsule is also greatly variable, although spore forming bacteria are rare in the rumen (Hungate, 1966). Microscopic studies have elucidated that the rumen microbial population is compartmentalised by feed particles with different populations associated with different particles and the rumen wall vs. the liquid phase. The distribution of these mobile populations is dictated by the action of the rumen wall as it relaxes and contracts, churning its contents, and as the animal regurgitates material (Sadet-Bourgeteau*et al.*, 2010).

Recent research suggests that there may be a 'core microbiome' shared between ruminants. Jami and Mizrahi (2012) characterised the bacterial populations of 16 lactating dairy cows using tag amplicon pyrosequencing. The study showed 82% similarity in bacterial taxa between the cows, with 32 genera shared by all animals – though with variation in abundance between samples. Overall, the bacterial populations of the sampled rumens appeared to be phylogenetically related, suggesting the functional requirements of the rumen have selected for bacteria in similar taxa (Jami and Mizrahi, 2012).

1.2.2: The rumen protozoa

Protozoa, along with fungi, are one of the two eukaryotic groups found in the rumen, and account for up to 50% of microbial biomass of the rumen (Belanche*et al.*, 2011). The majority of protozoa in the rumen are ciliates, with some parasitic flagellate species. Such flagellates are greatly in the minority and only a few genuine flagellates have been identified, for example *Trichomonas* spp., *Monocecromonas* spp. and *Chilomastix* spp., their metabolism remains relatively poorly understood (Williams and Coleman, 1997).

Figure 1.4: Rumen ciliates prepared using Lugol's solution (Martinele*et al.*, 2014). A-B: *Entodinium*sp; C: *Dasytricha*sp; D: *Isotricha*sp; E: *Enoploplastron*sp; F: *Eremoplastron*sp; G: *Eudiplodinium*sp; H: *Metadinium*sp; J: *Polyplastron*sp. Scale: 20µm. (Arrows indicate structural features)

The ciliate protozoa in the rumen are better understood and may be classified into two groups depending on their morphology: holotrichous or entodinomorphids, but may also be categorised according to their metabolism of various substrates (Fig 1.4) (Kamra, 2005). Holotrichs tend to utilise soluble carbohydrates and can be physically characterised by uniform length cilia covering the cell surface, with fused cilia around the vestibulum and flexible pellicles (Williams and Coleman, 1997; Dehority, 2003). The Entodiniomorphids, on the other hand display tufts of cilia located at the anterior or posterior end of the cell and may be classed using many features, such as ciliary zones, number of skeletal plates, caudal projections and the arrangement of cilia on the cell surface (Dehority, 2003). Entodinimorphids are responsible for the metabolism of a variety material, with certain species being capable of degrading plant cell walls via extracellular enzymes and ingestion of plant cells and cell wall fragments (Akin and Amos, 1978).

The two classes of protozoa display their own, independent diurnal cycles; Purser and Moir (1959) noted that Entodiniomorph concentrations decreased at feeding and then rose, while Purser (1961) later observed that holotrich concentrations peak at feeding time and then gradually diminish. Predominantly, the role of protozoa is to digest components of plant material (cellulose, hemicelluloses, fructosans, pectin, starch, insoluble sugars, proteins and lipids) which would otherwise be indigestible.

Protozoa are also known to ingest rumen bacteria, which are present in the digestive vacuoles and endoplasm; observations by Bryant and Small (1960) showed a doubling of rumen bacteria from 6.0 x 10^9bacteria/mL in calves inoculated with whole rumen contents, to 13.6 x 10^9 bacteria/mL in isolated calves. This highlights the considerable effect that protozoa have on the bacterial population, with some protozoa fully digesting bacteria, releasing Hydrogen (H_2) which could contribute towards CH_4 production and release (Williams, 1986; Morgavi*et al.*, 2010).

1.2.3: The rumen fungi

Anaerobic fungi form a minor portion of the microbial population in the rumen, but despite their smaller biomass, contribute significantly to overall metabolism due to high cellulolytic activity (Jenkins *et al.*, 2008).Many different strains of fungi have been reported in the rumen, populations varying between species of ruminant, this relates back to a previous point suggesting that each individual has a 'rumen fingerprint'. Species that have been isolated from the cattle rumen successfully include: *Neocallimastix*, *Caecomyces*, *Anaeromyces, Piromyces, Anaeromyces, Cyllamyces*and *Buwchfawromyces*.Of note, is that despite fungi being observed in the rumen as early as 1910, they were not reported until later on when it was uncovered that zoospores had been misidentified as flagellate protozoa (Fig 1.5). Recent research has suggested that the taxonomy of anaerobic rumen fungi may be much more complex than initially though, particularly in the phylum*Neocallimastigomycota*(Gruninger*et al.*, 2014). Using next generation sequencing, research aims to develop our understanding of the fungal life cycles with in the rumen, to take better advantage of the host of cellulolytic enzymes rumen fungi produce.

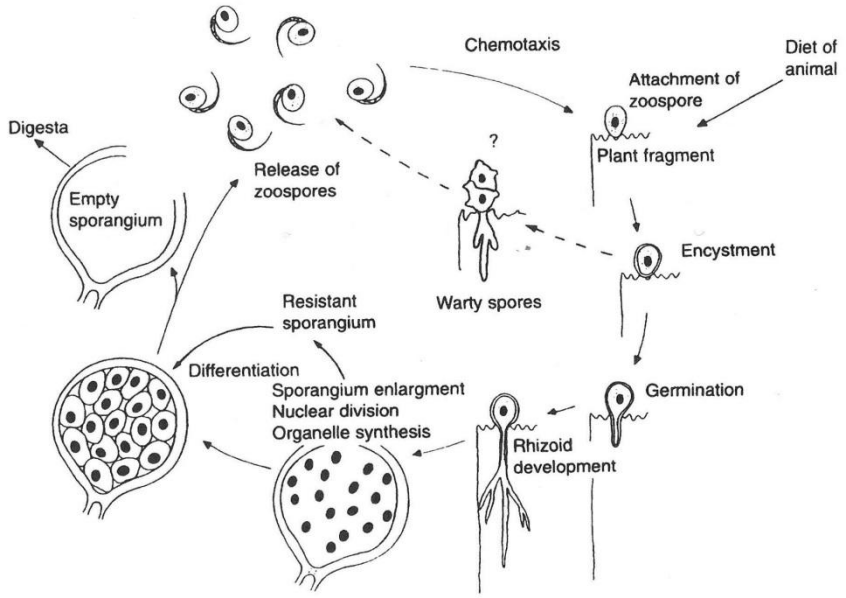


Figure 4.3 Schematic life cycle of monocentric species of anaerobic fungi.

Fig 1.5: Schematic life cycle of monocentric species of anaerobic fungi in the rumen (Orpin and Joblin, 1988).

Fungi play an important role in lignocellulose degradation, and are responsible for the release of enzymes such as α/β-galactosidases and hemicellulolytic glucosidases which allow rumen bacteria to further metabolise fatty acids (Williams and Orpin, 1987). The fungal life cycle is documented to last for 32 hor more and begins with attachment of motile zoospores, from the liquid phase, to lignified tissue (presumably located using chemotaxis). After attachment, zoospores will encyst and germinate to form a vegetative structure (comprised of the sporangium and rhizoid), it is then that the fungi will penetrate the food particle and begin digestion (Gordon and Phillips, 1998). Rumen fungi are relatively easy to manipulate via diet, and there is much evidence to support that fibre-rich forages or high-fibre concentrate diets stimulate fungal growth in the rumen (Kamra*et al.*, 2003).

1.2.4: The rumen Archaea

Members of archaea account for just 0.3-3.3% of the microbial population in the rumen, most of which are strict anaerobic methanogens. Most methanogen species utilise H_2 and formate as an energy source, and in the process use the electrons to reduce CO_2 to CH_4 – clearly implicating methanogens in the methane emissions in ruminants. Because of the importance of H_2 in fermentation, archaea are largely responsible for modulating rumen conditions and function. Similarly to the bacteria of the rumen, many archaea are ingested and associated with protozoa, almost all of which fall into the culturable genera. Culturable methanogens of the rumen have thus far been classified into one of five genera:

Methanobacterium, *Methanobrevibacter*, *Methanomicrobium*, *Methanoculleus* and *Methanosarcina*. *Methanobrevibacter*are considered the dominant methanogens (61%) although it is thought that around 40% of the total archaea in the rumen remain undiscovered (Shin *et al.*, 2004; Janssen and Kirs, 2008).

Whilst the methanogens constitute the majority of archaea in the rumen, they are not alone, and recent research has discovered several new species, completely unrelated to the methanogens. Shin *et al.*(2004) analysed the rumen ecosystem using 16S rDNA PCR from the bovine rumen, using solid, liquid or epithelial samples. Clones from the epithelium contained mainly (95%) known methanogens, but by contrast, 67% of clones from the liquid phase were unidentifiable along with 40% of clones from solid food particles. Interestingly, three clones contained rDNA related to non-thermophillic and thermophillic *Crenarchaeota* which have not been previously identified in the rumen. In a similar experiment by Tajima *et al.*(2001), 79% of clones from rumen fluid were unknown and distantly related to *Thermoplasma*aspp, in addition to the discovery of a new, novel cluster of Euryarchaeota.

Manipulation of methanogens for reducing CH_4 emissions is a challenging area, which holds much potential. Research has been focussed on vaccines, amongst other dietary strategies, which aim to decrease methane output by manipulating the methanogen population in the rumen. Williams *et al.*(2009) immunised 32 sheep with anti-methanogen vaccines specifically targeted to the most abundant species of the rumen, however there was no significant difference in methanogen population or methane output between the inoculated and control group, despite high specific Immunoglobulin (Ig) G concentrations. Wright *et al.* (2004) obtained, using highly specific antibody titres, a 7.7%/kg reduction in methane emissions of one group (however, upon replication, the study failed to achieve such a reduction). These studies highlight the difficulty of translating theory into application, although with further research it is possible that an effective methane mitigation vaccine could be produced.

1.3: Rumen lipid metabolism

Dietary lipids consist largely of phospho- and glycolipids, with some triglycerides being introduced by supplementation of the diets with oils. In terms of the fatty acids contained by these lipids, 18:2 linoleic acid (LA) and 18:3 linolenic acid (LNA) are most prevalent in ruminant nutrition, with oleic acid (18:1) also accounting for a large percentage of fatty acids derived from seed oils (Harfoot and Hazlewood, 1997).

1.3.1: Lipolysis

The process of fat breakdown begins with lipolysis, during which ester linkages of acyl lipids are hydrolysed to liberate free fatty acids, with a free carboxyl group, as required for the biohydrogenation process. Lipolysis also releases glycerol which is converted into volatile fatty acids (VFAs) which are absorbed into the blood (Harfoot and Hazlewood, 1997).

Lipolysis, as a process is driven mainly by bacterial lipases, although there is some contradictory evidence for the role of protozoal and fungal lipases, which does merit further investigation. One of the better known rumen lipasesis the extracellular lipase produced by *A. lipolytica*strain 5S (identified by Hungate, 1966), found at concentrations of 10^7/mL, it is able to rapidly hydrolyse triacylglycerides, and is therefore most active in ruminants fed supplements and concentrates (Henderson, 1971). Although the lipolytic capacity of *A. lipolytica* has been known since the 1970s, the lipases themselves have not been characterised until recently. Prive*et al.* (2014) identified 3 lipolytic genes from *A. lipolytica* strain 5ST,alipA, alipB and alipC. A and B showed homology to proteins of the Selenemonasspp and were categorised into the GDSL/SNGH family II, a group of esterases and lipases which show hydrolytic activity with a broad range of substrates. The GDSL/SNGH family displays little homology to the true lipases, with no nucleophillic elbow and a flexible active site (Akoh*et al.*, 2004). The third lipase, alipC shared a 42% identity with a lipase from a rumen metagenome and was categorised with the lipolytic enzymes of family V (Prive*et al.*, 2013). It is likely that more lipases exist within the genome of *A. lipolytica*but these may be uncharacterised genes in GenBank which makes sequence identification difficult; in addition, previous sequencing depth may not have been sufficient.As this enzyme belonging to *A. lipolytica*lacks the ability to hydrolyse phospho- and galactolipids, which account for a major portion of fats derived from grazing diets, this leaves a niche for *Butyrivibrio*-like species which produce numerous galactolipases and phospholipases (Harfoot and Hazelwood, 1997). Nonetheless, these data are based on pure culture studies which possess relatively narrow scope when it is considered that most rumen bacteria remain uncultured, and probably contain an array of lipases.

Indeed, using functional metagenomics of the whole rumen microbiome, Liu *et al.* (2009) characterised two novel lipase genes from a rumen metagenomic library – RlipE1 and RlipE2. BLAST elucidated that RlipE1 possessed 90% similarity to a carboxylesterase from *Thermosinuscarboxydivorans*(phylum Firmicutes) and RlipE2 displayed a lower, 50% homology to other lipolytic enzymes. Both lipases showed high hydrolytic affinity to laurate (C12), palmitate (C16) and stearate (C18), introducing two interesting targets for manipulation of rumen metabolism (Prive*et al.*, 2014).

Despite not directly contributing to the biohydrogenation process, protozoal lipolysis accounts for 30-50% of lipolytic activity in the rumen, with 75% of microbial fatty acids resulting from protozoal lipolysis – in particular vaccenic acid and conjugated linoleic acids (Devillard*et al.*, 2006). It is important to note that due to the fact protozoa ingest bacteria and that bacteria associate with the protozoal surface, it is very difficult to differentiate between bacterial and protozoal impact in the rumen (Williams and Coleman, 1997; Lourenco *et al.*, 2010).

There are also some studies on the salivary lipases of cows, although their input in lipolysis appears minimal. In suckling calves it has been established that lipolysis occurs before the milk reaches the stomach, leading to examination of the oral cavity which revealed lipase activity from glands at the back of the tongue and at the pharyngeal end of the oesophagus, releasing short chain FAs such as butyric acid (Edwards-Webb and Thompson, 1976). However this does not account for lipases in adult bovine saliva, which are arguably of more importance in industry.

Plant lipases and other compounds such as green leaf volatiles also contribute to the lipid metabolism of the rumen; plant tissues are rich in naturally occurring galacto- and phospholipases which remain active for up to 5 h after reaching the rumen. Recent studies have confirmed plant lipase activity, reporting increased free FA presence and reduced concentrations of polar lipids after incubation (Lee *et al.*, 2004; Lourenco *et al.*, 2010). Van Ranst*et al.* (2009) additionally reported 60.4% lipase activity of polyphenol oxidase of red clover, after incubation for 8 h, suggesting that plant lipases could play a larger role in ruminal lipid metabolism than initially thought.

1.3.2: Biohydrogenation

Released polyunsaturated fatty acids undergo the complex process of biohydrogenation which results in their conversion to SFAs. The metabolic pathways for both linoleic and linolenic acid are very similar, although the increased number of double bonds in LNA makes the process more complex (Lourenco *et al.*, 2010). LA metabolism begins with temporary formation of the intermediate conjugated linoleic acid (CLA), a highly beneficial fatty acid in human health which has been shown to act as an anti-carcinogenic, reduce risk of cardiovascular diseases (CVDs), as well as inhibiting lipogenesis in milk (Shingfield and Griinari, 2007). CLA as an intermediate arises in several different isomers, although the *cis*-9, *trans*-11 18:2 is most common, and due to health implications is better researched. As such it has been discovered that when feeding a diet high in fish or plant oil, CLA and oleic acid will accumulate in the rumen, leading to a decrease in milk lipogenesis (Bauman and

Griinari, 2001). LA may also be converted to rumenic acid (*cis*-9, *trans*-11 18:2 CLA), both of which are then converted to vaccenic acid (VA) and finally stearic acid (Lourenco *et al.*, 2010) (Fig 1.6). LNA metabolism differs in the number of potential intermediates that can be formed, the most common being *cis*-9, *trans*-11, *cis*-15 18:3 conjugated triene (Wasowska*et al.*, 2006), which is thought to have similar health benefits to CLA (Fig 1.6).

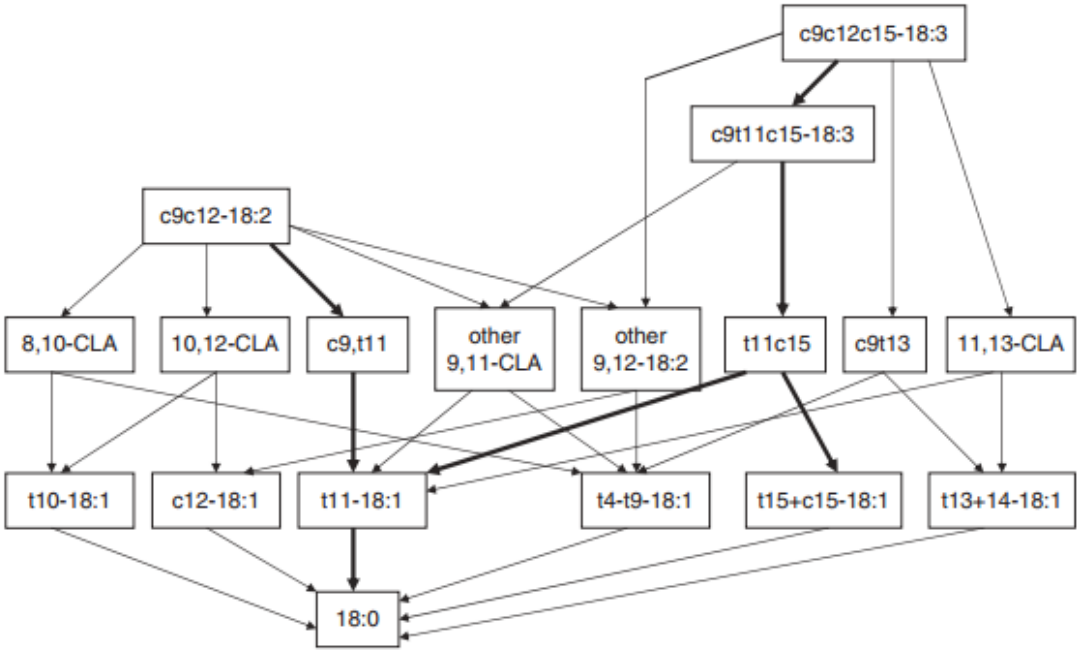


Fig1.6: Biohydrogenation pathways in the rumen. LA – c9c12-18:2; LNA – c9c12c15-18:3; VA- t11-18:1. CLA= Conjugatedlinoleicacid. LNA = Linoleicacid. VA = vaccenic acid. AdaptedfromChilliard*et al.* (2007).

The majority of biohydrogenation activity was previously attributed to the rumen bacteria, which can mostly be categorised into the *Butyrivibrio, Psudeobutyrivibrio* and *Clostridia* genera (Lourenco*et al.*, 2010). Recent research using next generation sequencing and advanced culturing techniques has shown biohydrogenating potential in other genera of bacteria (Janssen and Kirs, 2008; Huws *et al.,* 2014). Indeed, previously uncultured bacteria belonging to the genera: *Prevotella*, *Lachnospiraceae*, *Bacteroidales*, *Clostridiales* and*Ruminococcaceae,* have been identified as having biohydrogenating potential (Huws *et al.*, 2011; 2014).

A study by Nam and Garnsworthy (2007) found that fungi biohydrogenate linoleic acid, although when compared to bacteria the process occurs at a much slower rate (24 h vs. 1.5 h respectively). During the study, 18:2 linoleic acid was converted to 18:0 stearic acid by mixed rumen bacteria, while samples of mixed rumen fungi converted the linoleic acid to

18:1 vaccenic acid, with conjugated-linoleic acid as an intermediate. There is little research on fungi as biohydrogenators, however further study may be beneficial in terms of increasing concentrations of unsaturated fatty acids and reducing SFAs in ruminant products.

1.3.3: Manipulating ruminal lipid metabolism

Within the past 10 to 15 years, research has been directed into manipulating the process of biohydrogenation in the rumen as a means of reducing SFA content in meat and milk. Approaches generally include dietary manipulation, enzymatic manipulation through an alteration to the microbial population in the rumen. In light of the potential anti-microbial effects of PUFA, lipid supplements such as linseed, soyabean and sunflower seeds which are high in PUFA are fed to reduce SFA content in milk and meat. It is also true that these oil supplements are rich in PUFA, and have been fed to increase flow to the rumen, but ultimately the biohydrogenation capacity of the rumen bacteria results in little increases in flow to the duodenum. Sunflower and soyabean oils have been the most efficient in increasing levels of CLA in milk, whilst linseed and sunflower oils produced a higher level of *trans*-18:1 FA (Glasser*et al.*, 2008). A study by Chilliard*et al.*(2009) showed that feeding linseed oil caused a shift in the FA composition of milk, from 4:0 to 16:0 FAs to a higher concentration of 18:0, 16:1, 18:1, 18:0*, trans*-11 16:1, all *cis*- and *trans*-18:1 and non-conjugated *trans*-18:2 fatty acid isomers.

Fish oils have also been used as supplements with some success, a study by Lee *et al.* (2005), found that feeding a higher concentration of fish oil (herring offal) had no effect on the rumen pH or ammonia-nitrogen concentration, but did significantly reduce the total concentrations of VFAs. The flow of long chain PUFA, CLA and VA to the duodenum also increased considerably, while flow of oleic and linoleic acid remained unaffected. It was concluded that the fish oil inhibited the conversion of vaccenic acid to stearic acid, causing a build-up of the intermediate. A similar study by Kim *et al.* (2008) found that steers fed the highest concentration of fish oil (*Salmosalar*) had a 100% increase in *trans*-18:1 duodenal flow, compared to 39% in controls. 16S rRNA-based DGGE revealed significant changes in the rumen bacteria in the steers fed fish oil, but was unable to provide evidence that *Clostridium proteoclastium* plays a role in SFA/PUFA absorption.
It has long been established that feeding unprotected lipid supplements can reduce methane output, as PUFAs provide an alternative to methane for hydrogen attachment, and subsequently disposal. Reductions of up to 40% have been achieved, although 20-25% is more realistic (Beauchemin*et al.*, 2008). It has also been documented that oils are much more effective than seeds in reducing methane emissions and increasing duodenal PUFA

flow, with sunflower oil, myristic acid and soybean oil proving the most efficient in terms of % fed vs. effect (Beauchemin*et al.*, 2008).

Another, more controversial approach to inhibiting biohydrogenation is defaunation of the rumen; a study by Yanez-Ruiz *et al.*(2007) found that the rumen of protozoa free lambshad a lower PUFA/SFA ratio compared to controls and larger concentrations of CLA isomers. Protozoa-free lambs possessed higher tissue levels of *trans*-10, *cis*-9, *cis*- 12 – 18:2 amongst other PUFA compared to controls. Whilst the method is effective, ethical consideration must be taken into account when choosing a method of defaunation, with chemical methods being a more dangerous option due to toxicity levels. Emptying and treating rumen fluid before reintroduction can also be risky and it is also difficult to achieve replicable results. Isolation after birth is potentially one of the more reliable methods; whilst this is common practise on dairy farms, the ethical concerns about separating young from their dam must be taken into consideration (Jouany, 1991).

1.4: Study Aims

Maia *et al.*(2007) found that the presence of PUFA (50µg/mL or over) successfully inhibited biohydrogenation. Bearing this in mind it is hypothesized that increasing lipolysis may be an effective method of releasing more, free PUFA that will inhibit biohydrogenation, resulting in more PUFA flowing to the duodenum. Little research has been conducted into the process of lipolysis as a point for manipulating ruminal lipid metabolism. As such, this study aims to delve further into the process of lipolysis, and the way in which it could be altered to effect composition of ruminant products.

The study by Maia *et al*. (2007) tested the toxicity of PUFA towards *Butyrivibrio* spp., nonetheless recent data suggest that other bacteria may play a more predominant role in rumen biohydrogenation (Janssen and Kirs, 2008; Huws *et al.*,2014). This brings into question the true levels of PUFA required to be toxic to most biohydrogenating bacteria. Thus the primary aim of this study was to evaluate the PUFA concentrations required to inhibit the biohydrogenation capacity within the whole microbiome. Secondly, we evaluated the possibility of achieving levels of PUFA identified in the above study through increasing lipolysis by the addition of various levels of phospholipases to *in vitro* incubations containing rumen fluid and extracted perennial ryegrass phospholipids.

2: Assessing the levels of Linoleic Acid and Linolenic Acid required for inhibition of biohydrogenation

2.1: Introduction

It is believed that the primary aim of biohydrogenation in the rumen is to hydrogenate toxic polyunsaturated fatty acids, liberated during lipolysis, into harmless fatty acids. The rumen microbes do this to avoid the hypothesised bacteriostatic effects of polyunsaturated fatty acids, and the significant expenditure of cellular resources due to biohydrogenation highlight it's importance (Maia *et al.*, 2010). As previously mentioned in the literature review, the biohydrogenation process is a complex metabolic process, with several intermediates including conjugated linoleic acid (CLA; 18:2, *cis*-9, *trans*-11), a human health beneficial fatty acid. Nonetheless, the end products of biohydrogenation are human health detrimental saturated fatty acids. Thus if biohydrogenation can be controlled, or inhibited, a higher concentration of unsaturated fats (and potentially CLA) would flow to the duodenum and be available for incorporation into meat and milk, whilst decreasing theircontent of saturated fats. Theories as to why unsaturated fats may be toxic to rumen bacteria are varied, and as yet no definite mechanism has been elucidated. The most possible explanation is that the double bonds in PUFA which alter the structure of the molecule cause a 'kink' which if incorporated into the lipid bilayer of bacteria will disrupt the membrane, causing leakiness and compromising cell integrity (Maia *et al.*, 2010). However research by Maia *et al.* (2010) found that there was no difference in propidium Iodide ingression (as used to assess cell integrity) between PUFAs with fewer double bonds and those with a higher number. This evidence suggests that the toxicity of PUFA is perhaps not concerned with the membrane, but maybe due to the diffusion of free fatty acids across the membrane, which then causes chemiosmotic problems such as ion leakage (Nicholls *et al.*, 1982).

Relatively little research has been directed into the toxicity of different types and concentrations PUFAs to rumen microorganisms. One notable study by Maia *et al.*(2007) investigated the effects of Linoleic Acid (LA; *cis*-9, *cis*-12–18:2), Linolenic Acid (LNA; *cis*-9, *cis*-12, *cis*-15–18:3) and two other fish oil fatty acids (eicosapentaenoic acid 20:5 n-3 and docosahexaenoic acid 22:6 n-3) on pure cultures of rumen bacteria and fungi. Maia *et al.* (2007) assessed the lipid metabolism and growth of 26 strains of bacteria and two species of fungi when inoculated with 50 $\mu g/mL^{-1}$ and 20 $\mu g/mL^{-1}$ of LA. Growth and metabolism were inhibited in 5 of the tested strains, of which three were butyrate producers and two were cellulolytic*Ruminococcus* sp. When the concentration of LA was reduced to 20 $\mu g/mL^{-1}$, only the two previously mentioned *Ruminococcus* species were inhibited. Other strains

(*Anaerovibriolipolytica*, *Butryvibriospp (*not *B. hungatei), Clostridium proteoclasticum*B316^T *and* P-18*, Eubacteriumpyruvativorans, Fibrobacter succinogenes, Lachnospira multipara, Megaspheraspp, Mitsuokellamultiacidus,Peptostreptococcusanaerobius, Prevotellaspp, Ruminobacteramylophilus, Selenomonasruminantium, Streptococcus bovis and Veilonellaparvula)* were able to metabolise LA at such a concentration, with varying end products, the most common of which being Vaccenic acid, with only *Clostridium proteoclasticum*B316^T forming *cis*-9, *trans*-11–18:2. The study also ranked fatty acids in terms of toxicity to growth: Eicosapentaenoic acid >Docosahexaenoic acid > α-Linolenic acid > Linolenic acid. As would be expected, when the concentration of LA was increased to 200 μg/mL^{-1} cell density decreased, suggesting that cell growth and integrity (and perhaps lipid metabolism) can be reduced upon addition of higher concentrations of PUFA. This study leaves an open question of whether higher concentrations of PUFAs (such and LNA or LA) could compromise cell growth and integrity to such a point that biohydrogenation is reduced within the animal itself.

A later experiment by Maia *et al.* (2010) explored the toxicity of unsaturated fatty acids to *Butryvibiofibrisolvens*. This study used several PUFAs, including linoleic and linolenic acid, and measured cell integrity using propidium iodide, fatty acid profiles after incubation with LA/LNA, and metabolic effects using Acyl CoA. In keeping with a previous study by Kim *et al.* (2000) the study found that the effect of LA depended not just upon its concentration but also upon the growth stage of bacteria – that is, growing bacteria were more tolerant. Alongside cellulolytic species, butyrate forming bacteria consistently show more sensitivity to PUFA compared to other non-butyrate forming bacteria, but those with the butyrate kinase system appear more vulnerable than those using the acyl transferase mechanism (Fukuda, 2005). Meanwhile, the most tolerant strains had the highest linoleate isomerase activity, responsible for the conversion of CLA to LA (Fukuda, 2005). This study highlighted our need to pinpoint exactly how and where PUFA act and the toxicity of the chosen PUFA, linoleic and linolenic acid, as well as drawing distinctions between the sensitivity of butyrate producers and non-butyrate producers. Whilst this experiment provided novel information regarding the toxicity of PUFA to *B. fibrisolvens*, the effect upon the rumen microbiome remains unaddressed, as such, any application to the animal and its products is limited.

As previously indicated, the rumen bacteria are mostly responsible for biohydrogenation, with some input from fungi; however culturable isolates only account for approximately 11% of the microbial population suggesting experiments using only pure cultures are possibly limited in terms of interpreting biological relevance within the rumen itself (Edwards *et al.*, 2004, Ross *et al.*, 2012). In contrast to other experiments, in this study we utilised a mixed population from fresh rumen fluid. Using a mixed population in rumen

fluid allows a more accurate simulation of the natural rumen environment and may also include the activity of uncultured species.

2.2 Materials and methods

The experiment was a simple batch culture experiment with six treatments, each with the appropriate amounts of anaerobic buffer, fresh rumen fluid (50%) and either: control (No LA/LNA) or LA/LNA at a varying concentration. Two sample groups: LA and LNA, both had the appropriate fatty acid added at: 50 µM and 250 µM, 500 µM, 750 µm and 1mM, along with a control. Each treatment had three replicate incubations per each of the three time points (0, 4, 8 and 24 h). Total number of samples was 144.

2.2.1: Rumen inoculum

Three non-lactating, rumen-cannulated Holstein-Friesian dairy cows each provided 0.5 L of hand squeezed and strained rumen fluid. Cows are located at Aberystwyth University's Trawsgoed Experimental Farm (Aberystwyth, Wales) and at the time were fed premium and high-sugar perennial rye grass silage with minimal grazing, and free access to water. Sampling was carried out at approximately 8:30am, 1.5 h after feeding. Samples were pooled before transportation to the lab in thermo flasks, upon arrival flasks were transferred to an incubator at 39° C.

2.2.2: Preparation of buffer

2 L of buffer was prepared the day before each experiment, according to Goering and Van Soest (1970) (Table 2.1 and 2.2):

Table 2.1: Guidelines for the preparation of each component required for Van Soest buffer (Goering and Van Soest, 1970).

		g/L dH_2O
Microminerals	Calcium chloride2-hydrate	13.2
	Manganese chloride4-hydrate	10
	Cobalt chloride6-hydrate	1
	Ferric chloride6- hydrate	8
Buffer	Sodium hydrogen carbonate	39.25

Macrominerals	Di-sodium hydrogen orthophosphate 12-hydrate	9.45
	Potassium di-hydrogen orthophosphate (anhydrous)	6.2
	Magnesium sulphate 7-hydrate	0.6
Reducing Agent	dH_2O	200 mL
	Cystein hydrochloride	5
	1 Molar Sodium Hydroxide	4 mL

Table 2.2: Guidelines for the preparation of 2 litres of Van Soest buffer

Constituent	Amount added
dH_2O	1.1 L
Buffer	0.44 L
Macrominerals	0.44 L
Reducing Agent	80 mL
Resazurin (0.1% w/v)	2 mL
Microminerals	0.2 mL

2.2.3: Preparation of LNA and LA

Linolenic acid ($C_{18}H_{30}O_2$; ≥99% purity) of Molecular weight 278.43 and Linoleic acid ($C_{18}H_{32}O_2$; ≥99% purity) of Molecular weight 280.50 was obtained from Sigma Aldrich (Dorset, UK). Both fatty acids (139 µl) were diluted in 10 mL of Van Soest buffer to achieve a 50 mM concentration. Mixtures were vortexed and heated briefly at 39°C to ensure thorough mixing and acclimatisation to rumen-like temperatures.

2.2.4: *In vitro* experiments

Van Soest buffer (Table 2) was added to 144 hungate tubes, the volume of which was dependent upon on the amount of LNA/LA to be added (Table 2.3):

Table 2.3: The preparations of each Hungate tube according to the volume of fatty acid to be added.

Van Soest buffer (mL)	LA/LNA (µL)	Rumen fluid (mL)	Final concentration (µM)
4.99	10	5	50
4.75	25	5	250
4.50	50	5	500
4.25	75	5	750
4.0	100	5	1000
5.0	N/A	5	0 (Control)

All Hungate tubes containing buffer were autoclaved (Astell, Benchtop Autoclave) for approximately 20 mins at 121°C, to sterilise and provide an anaerobic environment, and then incubated at 39°C, to mininmise temperature disruption to microorganisms upon addition.
LA/LNA and rumen fluid (50%) was then added at the appropriate volumes to achieve the desired final concentrations, with continual CO_2 purging (Table 2.3).

All tubes aside from 0 h samples were then incubated in the dark at 39°C. Note no LA and LNA were added to 0 h samples.

2.2.5: Destructive harvesting

Samples were harvested by vigorous vortexing and a 200 µL aliquot taken for RNA extraction before retaining the remainder in the freezer at -20°C. The remaining sample was frozen and then freeze dried in preparation for lipid analysis.

0 h samples were taken promptly, after addition of rumen fluid and LNA/LA.

2.2.6: Fatty acid methylation

Freeze dried samples were homogenised (ground) and then weighed out to approximately 0.25 g/250 mg (Appendix CD). Heptane (1 mL) with 1.0 mg/mL of internal standard C21ME was added to each test tube along with 4 mL 0.5 M sodium methoxide/methanol (5 g dry sodium hydroxide in 250 mL anhydrous methanol). Tubes were hand mixed and heated at 50°C in a water bath for 15 minutes, then removed; hand mixed again and allowed to cool. Then, 4 mL of acetyl chloride/methanol (Acetyl chloride: Methanol, 1:10) on ice was added and the tubes heated in a water bath to 60°C for 1 h, with hand mixing occasionally. A further 2 mL of heptane and 2 mL distilled water was added, the

tubes were vortexed thoroughly for approximately 5 secs and then centrifuged at 2000 rpm for 5 mins.

Supernatant was then removed from the top layer using a Pasteur pipette, into gas chromatography vials which were frozen at -20°C for gas chromatography analysis.

2.2.7: Fatty acid analysis

The Fatty Acid Methyl Esters (FAME) were analysed using a Varian CP-3800 GC-FID on an Agilent CP-Select for Fame column (100m X 0.25mm ID, Varian Inc.) using Helium flowing at 1.5 mL/min. Thermal ramping was set from 70°C to 220°C to maximise isomer separation. The injector was maintained at 250°C, to promote even volatilisation, and the flame ionisation detector at 255°C.

2.2.7: RNA extraction and quantification

Frozen 200 µl samples were thawed and a 100 µl aliquot taken for RNA extraction, the remaining 100 µl was placed back at -80°C for long term storage.

A FastRNA Pro Soil-Direct kit from MP Biomedicals (UK) was used to extract RNA from samples, following the manufacturer's guidelines.

Once extracted, 1 µl of each sample was used to quantify approximate RNA concentration in duplicate on an Epoch Micro-Volume Spectrophotometer System from BioTek (US) using Gen5-Take3 software. Samples were run using the dsDNA and Microdot setting on a Take3 plate to quantify RNA content. After quantification, data was exported to Microsoft Excel and samples were stored at -80°C overnight.

2.2.8: DNase treatment and Reverse transcription

To remove any contaminating DNA from the RNA extractions prior to RT-PCR RQ1 RNase-Free DNase kit (Promega, UK) was used, following manufacturer's guidelines with 4 µL of RNA.

PCR was then carried out to ensure the absence of contaminating DNA. Primers 27F and 1389R (Table 2.4) were diluted to 1:3 using 25 µL and 75 µL of PCR grade water to achieve a final concentration of 250µM. A master mix was created containing (per sample): 12.5 µL MyTaqRedmix 2X (Bioline), 0.5 µL of diluted forward and reverse primers and 9.5 µL

of PCR grade water. 23 µL of master mix was then divided into the appropriate number of aliquots and 2 µL of DNase treated RNA added. The PCR amplification was then carried out using an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Warrington, UK) for 2.5 h. The following conditions were used for 25 cycles: 95°C for 2 minutes (first cycle only), 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 1 minute, 72°C for 7 minutes (final cycle only). Samples were then held at 4°C until removal.

2.4: PCR primers and sequences used in DNase treatment and cDNA synthesis.

Primer	Sequence
27F	5'-AGAGTTTGATCCTGGCTCAG-3'
1389R	5'-ACGGGCGGTGTGTACAAG-3′

Samples (5µL) were loaded onto a 1% agarose gel, with a 1 Kb ladder and run at 120 V for 30 mins. The gel was visualised on a trans-UV setting using Quantity One software and Molecular Imager®GelDoc™XR+ System (Bio-Rad, UK). The image was then visually assessed for the presence of banding.

Samples for LA remained contaminated despite DNase treatment, so Lithium Chloride precipitation was also used. RNA (5 µL) was added to 45 µL of DEPC treated water and 25 µL of cold 2.5 M Lithium Chloride was added, then samples were mixed and left for 48 h at 4°C. Samples were then centrifuged at 4°C for 30 minutes at 13,000 rpm, the supernatant discarded and a further 25 µL of cold 2.5 M LiCl added before vortexing. Samples were centrifuged again and the same volume ofLiCl added before vortexing and discarding the supernatant. 50 µL of cold 80% ethanol was added to clean the RNA pellet and samples vortexed before being centrifuged at 4°C for 30 minutes at 13,000 rpm and supernatant discarded. The cleaning procedure was repeated, and then a PCR and gel electrophoresis was performed and visualised, as previously described.

Once samples were free of DNA, reverse transcription was performed using 4 µL of primer 1389R in 196 µL of PCR grade water. 'Mix A' was prepared using 100 µL of dNTPs and 100 µL of diluted 1389R primer, then 2 µL aliquots of the mix were divided into appropriately labelled tubes along with 3 µL of the corresponding DNA-free, RNA (Table 2.5). Samples were then heated at 70°C for 10 minutes and cooled to 4°C, before adding 7 µL of Superscript Master mix (Table 2.5) following the protocol in the SuperScript Reverse Transcriptase kit (Invitrogen, UK). Samples were then incubated at 55°C for 60 minutes, 70°C for 15 minutes and cooled to 4°C.

Table 2.5: Composition of the Superscript Master Mix using SuperScript III Reverse Transcriptase kit (Invitrogen, UK).

Reagent	Volume (µL)
5X First Strand Buffer	400
0.1M Dithiothreitol (DTT)	100
SuperScript III Reverse Transcriptase	100
PCR grade water	100

PCR was then carried out on all samples, as before using the same primers but the forward primer was labelled at the 5' end with 6-carboxyflourescin and stored at 4°C overnight before commencing gel electrophoresis on 1% agar gel to visually assess products.

Some samples did not show banding at the required level, so PCR and gel electrophoresis was repeated with 4 µL of fresh cDNA.

2.2.9: Gel clean up and Terminal restriction fragment length polymorphism

To remove unused primers and DNTPs from the PCR products, an Isolate II PCR and Gel kit (Bioline, UK) was used following manufacturer's instructions.

The PCR clean-up products were then diluted using PCR grade water to give a standardised concentration of 10 ng/µL.

The restriction digest was carried out using HaeIII (Promega, UK) and diluted cDNA (Table 2.6). The amount of cDNA used in the first run was 5 µL, however this did not give a high enough concentration overall for the TRFLP run, so 8.75 µL was used in the second run.

Table 2.6: Composition of HaeIII mix using the HaeIII kit components (Promega, UK).

Reagent	Volume per sample (µL)	Volume for 96 well plate (µL)
Buffer 10X	1	100
HaeIII	0.25	25
Water	3.75/0	375/0
cDNA	5/8.75	

The 96-well plate was placed in the thermocycler at 37°C for 5 h and 65°C for 10 minutes, and held at 4°C overnight.

As described by Huws *et al* (2011) the terminal fragments were separated by size on a Beckman CEQ8000 sequencer (Beckman Coulter, UK) using GenomeLab DNA size standard kit (Beckman Coulter, UK). Fragments were then aligned using the Beckman CEQ8000 software binning function and data exported into Microsoft Excel. Peaks less than 0.5% of the cumulative peak height were removed. Data were then imported into Bio-Rad fingerprinting software (Bio-Rad, UK) and clustering analysis performed using 0.5% optimisation and 0.5% band tolerance. The resulting data were visualised using GeneMapper 5 software (Life Technologies, UK).

2.10: Statistical analysis of fatty acid profiles

Using Genstat (13th Edition) an ANOVA was run to analyse the effects of LA/LNA concentration and time upon fatty acid composition, Duncan's multiple range test was also included to provide significance levels for the differences between concentrations.

2.3: Results

2.3.1: Fatty acid profiles

In the majority of samples, there was a significant change in saturated fatty acid (18:0) content and in PUFA content (18:2 *trans*-9, *trans*-11, 18:2 *cis*-9, *cis*-12 and 18:3 *n*-3) (Table 2.7 and 2.8). In most samples there was also a significant effect of both time and concentration of LNA/LA added on the fatty acid profile.

Specifically treatments with 250 µM, 500 µM and 750 µM LNA at 4 h show the greatest reduction in 18:0 content. A significant difference in 18:0 content compared with controls was achieved when using a 250 µM concentration at 4 h, with a reduction of 11.5% (52.3% to 40.8%), and of 16.7% (60.8% to 44.1%) using LNA (Table 2.7 and 2.8). Stearic acid (18:0) content was reduced by 10% when using 500 µM LNA and by 8.6% using LA, at 4 h (Table 2.7 and 2.8). Contrary to the expected pattern, the higher the concentration of PUFA added, the lower the reduction in 18:0. When 750 µM of LNA was added at 4 h, a reduction of 3.8% was observed, but there was a significant increase in 18:0 in the presence of 750 µM LA. Furthermore, there was very little difference between controls and samples with 1 mM LA/LNA added throughout all time periods, with the most notable reduction being that of 7% using LNA at 4 h (Table 2.7 and 2.8). Similarly, there was little change following inclusion of

50 µM LA/LNA with samples at 0 and 24 h showing higher 18:0 content (50 µM LNA: 54.9% to 59.2% after 24 h). After 24 h the difference between treated samples and controls was much less apparent. Controls for LA at 24 h contained 5.8% more 18:0 than 24 h samples, and when using LNA 18:0 content increased by 2.9% between controls and 24 h samples (250 µM LNA) (Table 2.7 and 2.8). There is a clear distinction between the 18:0 content of samples at 0 and 24 h, and those at 4 and 8 h, with 18:0 content dipping with 250 µM and 500 µM LA/LNA at 4 and 8 h, and peaking with 1 mM concentrations over all time periods (Table 2.7 and table 2.8).

Regarding changes in the 18:1 *trans*-11 content of samples, a similar pattern appears as above. The largest increase occurs at 4 h with 250 µM LNA where the concentration increases by 3.48% and with 50 µM an increase of 1.66% (Tables 2.7 and 2.8). On the other hand, there were fewer significant changes in 18:1 *trans*-11 content when LA was added.

18:2 *cis*-9, *cis*-12 appears to show very little to no response to addition of either LA or LNA, with elevations in concentration being minimal (0.03% at their most extreme). In some cases there was a decrease between controls (0.07%) and 4 h samples (0.02%). However in terms of 18:2 *cis*-9, *cis*-11 there was more considerable changes. At 4 h the concentration of 18:2 *cis* -9, *cis*-11 raised from 0.53 to 1.28% after addition of 750 µM LNA, and from 0.38 to 1.58% with 50 µM LNA. More significant increases were observed upon addition of LA with the most increases observed in 4 h samples. At 4 h, inclusion of 250 µM LA caused an increase of 1.3% 18:2 *cis*-9, *cis*-11. After 24 h, again the most notable change occurred in tubes treated with 250 µM LA (18:2 *cis*-9, *cis*-12 concentration was 1.04%) and the rest of the group decreased slightly compared to samples at 8 h (Table 2.7).

With regards to changes in 18:3 *n*-3 (α-Linolenic acid) content, LNA did not elicit as significant a response as LA (Table 2.7 and 2.8). When using 50 µM LNA at 4 h 18:3 *n*-3 content rose by 0.85%, and by 0.25% when using 250 µM. There was a slight rise when using other concentrations of LNA, but not of a significant amount. At 8 h 250 µM LNA caused a rise in 18:3 *n*-3 to 0.80%and with 500 µM LNA 1.46% of 18:3 *n*-3 was detected (Table 2.7 and 2.8). When using LA, the difference between controls and treated samples was more significant. At 4 h the concentration of 18:3 *n*-3 increased by 1.6% using 250 µM LA– 18:3 *n*-3 content of samples at 4 h between 50 µm and 750 µM LA treatments was above 0.84%.

Of note, is that the expected dramatic increase in PUFA content upon addition of LA/LNA is not evident here. That is because 0 h samples contained no LA/LNA, so by the time samples were taken for 4 h, the majority of 18:2, *cis*-9, *cis*-12 and 18:3 *n*-3 have been biohydrogenated.

There was no significant difference between the addition of LA/LNA and long chain PUFA (LCPUFA) content between 0 and 4 h, however there was a significant decrease in LCPUFA at 24 h for samples containing 750 µM LA (1.92% to 1.17%) (Appendix CD).

Overall, LA/LNA addition caused no change in branched and odd chain PUFA (BOCs).

Table 2.7: Fatty acid profiles (as percentage of total fatty acids) of samples treated with different concentrations of linoleic acid (0 µM, 50 µM, 250 µM, 500 µM, 750 µM and 1 mM) incubated up to 24 h.

linolenic acid									
Time	**Fatty Acid**							**SED**	**P**
		0	**50**	**250**	**500**	**750**	**1000**		
0 h	12:0	0.37^{a}	0.45^{a}	0.29^{a}	0.32^{a}	0.39^{a}	0.31^{a}	0.12	0.737
	14:0	1.87^{a}	1.40^{a}	1.31^{a}	1.67^{a}	1.87^{a}	1.30^{a}	0.36	0.403
	16:0	18.22^{ab}	17.40^{ab}	14.89^{a}	15.95^{ab}	19.20^{b}	16.07^{ab}	1.56	0.131
	18:0	54.9^{a}	59.0^{a}	60.8^{a}	51.6^{a}	52.5^{a}	60.9^{a}	5.20	0.335
	18:1 *trans*-11	4.16^{a}	3.86^{a}	4.13^{a}	6.65^{b}	4.08^{a}	4.56^{a}	0.51	0.001
	Sum of 18:1 *trans*	5.03^{a}	4.68^{a}	5.23^{a}	8.29^{b}	5.09^{a}	5.70^{a}	0.60	<.001
	Sum of 18:1 *cis*	0.89^{ab}	0.75^{a}	0.87^{ab}	1.10^{c}	0.98^{bc}	0.90^{ab}	0.07	0.005
	18:2 *cis*-9, *cis*-12	0.06^{a}	0.06^{a}	0.05^{a}	0.07^{a}	0.06^{a}	0.07^{a}	0.01	0.418
	18:2 *cis*-9, *trans*-11	0.61^{ab}	0.38^{a}	0.83^{bc}	1.05^{c}	0.72^{abc}	0.52^{ab}	0.19	0.047
	18:3 *n*-3	0.66^{a}	0.48^{a}	0.67^{a}	1.45^{b}	0.74^{a}	0.70^{a}	0.32	0.123
4 h	12:0	0.31^{a}	0.37^{a}	0.46^{ab}	0.54^{b}	0.33^{a}	0.42^{ab}	0.06	0.034
	14:0	1.37^{a}	1.80^{ab}	2.03^{ab}	2.55^{b}	1.80^{ab}	2.00^{ab}	0.36	0.106
	16:0	16.51^{a}	18.23^{a}	19.36^{a}	18.89^{a}	19.83^{a}	17.79^{a}	2.85	0.870
	18:0	58.9^{b}	49.8^{ab}	44.1^{a}	41.6^{a}	48.7^{ab}	51.9^{ab}	6.19	0.162
	18:1 *trans*-11	5.48^{ab}	5.52^{ab}	7.61^{b}	6.33^{ab}	5.55^{ab}	3.64^{a}	1.30	0.151
	Sum of 18:1 *trans*	6.56^{ab}	6.68^{ab}	9.11^{b}	7.68^{ab}	6.63^{ab}	4.56^{a}	1.46	0.135
	Sum of 18:1 *cis*	0.90^{a}	0.97^{a}	1.05^{a}	0.99^{a}	0.94^{a}	0.91^{a}	0.08	0.462
	18:2 *cis*-9, *cis*-12	0.06^{bc}	0.08^{c}	0.07^{bc}	0.02^{a}	0.06^{bc}	0.03^{ab}	0.02	0.021
	18:2 *cis*-9, *trans*-11	0.53^{a}	1.580^{a}	0.76^{a}	0.80^{a}	1.28^{a}	0.57^{a}	0.54	0.360
	18:3 *n*-3	0.70^{a}	1.51^{b}	0.91^{abc}	0.84^{a}	0.90^{ab}	0.64^{a}	0.27	0.075
8 h	12:0	0.50^{a}	0.62^{ab}	0.76^{b}	0.65^{ab}	0.51^{ab}	0.49^{a}	0.11	0.154
	14:0	1.30^{a}	1.51^{a}	2.21^{b}	1.67^{b}	1.57^{ab}	1.42^{a}	0.29	0.090
	16:0	18.45^{a}	18.53^{a}	22.62^{b}	18.22^{a}	16.15^{a}	16.52^{a}	1.05	<.001
	18:0	57.0^{b}	53.8^{b}	42.9^{a}	50.1^{ab}	55.6^{b}	57.4^{b}	3.61	0.013
	18:1 *trans*-11	3.54^{a}	3.37^{a}	3.89^{a}	3.98^{a}	4.84^{a}	3.59^{a}	0.71	0.402
	Sum of 18:1 *trans*	4.30^{a}	4.16^{a}	4.73^{a}	4.80^{a}	5.90^{a}	4.37^{a}	0.81	0.358
	Sum of 18:1 *cis*	0.75^{a}	0.78^{ab}	0.90^{bc}	0.98^{c}	0.90^{bc}	0.83^{ab}	0.06	0.023
	18:2 *cis*-9, *cis*-12	0.05^{b}	0.05^{b}	0.00^{a}	0.07^{c}	0.08^{c}	0.08^{c}	0.01	<.001

	18:2 *cis*-9, *trans*-11	0.44^{ab}	0.42^{a}	0.64^{abc}	1.13^{ac}	0.44^{ab}	0.43^{a}	0.29	0.185
	18:3 *n*-3	0.76^{a}	0.71a	0.80^{a}	1.46^{b}	0.67^{a}	0.67^{a}	0.29	0.116
24 h	12:0	0.57^{a}	0.38^{a}	0.41^{a}	0.41^{a}	0.56^{a}	0.44^{a}	0.09	0.198
	14:0	1.62^{a}	1.53^{a}	1.64^{a}	1.62^{a}	2.18^{a}	1.94^{a}	0.29	0.252
	16:0	17.47^{ab}	16.62^{a}	16.86^{a}	14.91^{a}	17.65^{ab}	21.20^{b}	1.71	0.059
	18:0	54.9^{a}	59.2^{a}	57.8^{a}	55.2^{a}	49.4^{a}	49.4^{a}	4.80	0.263
	18:1 *trans*-11	3.43^{a}	3.65^{a}	4.05^{a}	4.47^{a}	4.56^{a}	4.12^{a}	0.67	0.531
	Sum of 18:1 *trans*	4.23^{a}	4.51^{a}	4.98^{a}	5.93^{a}	5.56^{a}	5.10^{a}	0.82	0.368
	Sum of 18:1 *cis*	0.82^{ab}	0.80^{a}	0.78^{a}	0.95^{b}	0.86^{ab}	0.77^{a}	0.06	0.063
	18:2 *cis*-9, *cis*-12	0.06^{a}	0.05^{a}	0.05^{a}	0.06^{a}	0.04^{a}	0.06^{a}	0.02	0.761
	18:2 *cis*-9, *trans*-11	0.41^{a}	0.46^{a}	0.49^{a}	1.26^{b}	0.50^{a}	0.47^{a}	0.14	<.001
	18:3 *n*-3	0.55^{ab}	0.52^{ab}	0.57^{ab}	0.64^{b}	0.50^{ab}	0.46^{a}	0.07	0.243

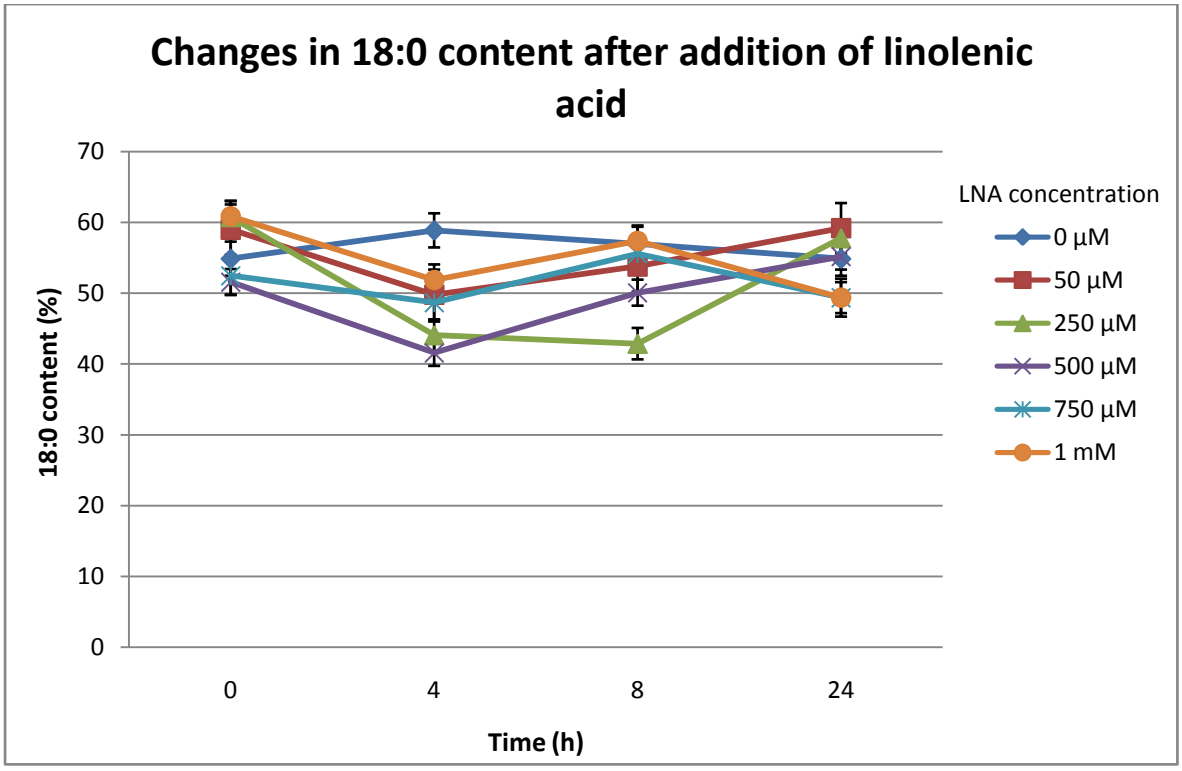
A two-way ANOVA was also conducted upon raw data for linoleic acid to assess the effect of different concentrations and incubation times on fatty acid profiles (Appendix CD). SED: Standard Error of Differences of means, P: significance of effect of LA concentration and incubation time (P=<0.05), ND= Not Detectable. Values within the same row were allocated different superscripts when significantly different.

Table 2.8: Fatty acid profiles (as percentage of total fatty acids) of samples treated with different concentrations of linoleic acid (0 µM, 50 µM, 250 µM, 500 µM, 750 µM and 1 mM) incubated up to 24 h**.**

linoleic acid									
Time	**Fatty Acid**	**Concentration (µM)**						**SED**	**P**
		0	**50**	**250**	**500**	**750**	**1000**		
0 h	12:0	0.37^{a}	0.45^{a}	0.29^{a}	0.32^{a}	0.39^{a}	0.31^{a}	0.12	0.737
	14:0	1.87^{a}	1.40^{a}	1.31^{a}	1.67^{a}	1.87^{a}	1.30^{a}	0.36	0.403
	16:0	18.22^{ab}	17.40^{ab}	14.89^{a}	15.95^{ab}	19.20^{b}	16.07^{ab}	1.56	0.131
	18:0	54.9^{a}	59.0^{a}	60.8^{a}	51.6^{a}	52.5^{a}	60.9^{a}	5.20	0.335
	18:1 *trans*-11	4.16^{a}	3.86^{a}	4.13^{a}	6.65^{b}	4.08^{a}	4.56^{a}	0.51	0.001
	Sum of 18:1 *trans*	5.03^{a}	4.68^{a}	5.23^{a}	8.29^{b}	5.09^{a}	5.70^{a}	0.60	<.001
	Sum of 18:1 *cis*	0.89^{ab}	0.75^{a}	0.87^{ab}	1.10^{c}	0.98^{bc}	0.90^{ab}	0.07	0.005
	18:2 *cis*-9, *cis*-12	0.61^{ab}	0.38^{a}	0.83^{bc}	1.05^{c}	0.72^{abc}	0.52^{ab}	0.19	0.047
	18:2 *cis*-9, *trans*-11	0.06^{a}	0.06^{a}	0.05^{a}	0.07^{a}	0.06^{a}	0.07^{a}	0.01	0.418
	18:3 *n*-3	0.66^{a}	0.48^{a}	0.67^{a}	1.45^{b}	0.74^{a}	0.70^{a}	0.32	0.123
4 h	12:0	0.45^{a}	0.59^{b}	0.79^{b}	0.82^{b}	0.63^{b}	0.68^{b}	0.11	0.051
	14:0	1.34^{a}	1.65^{b}	2.24^{b}	2.18^{b}	1.73^{b}	1.75^{b}	0.29	0.059
	16:0	17.3^{b}	17.8^{c}	19.4^{c}	17.8^{c}	16.5^{b}	15.7^{a}	0.75	0.006
	18:0	54.7^{b}	49.9^{b}	40.8^{a}	43.0^{a}	50.6^{b}	49.7^{b}	4.40	0.067
	18:1 *trans*-11	3.94^{b}	3.91^{b}	3.65^{b}	3.36^{a}	3.75^{b}	4.16^{b}	0.25	0.087
	Sum of 18:1 *trans*	4.75^{d}	4.97^{d}	4.50^{b}	4.19^{a}	4.64^{c}	5.12^{d}	0.18	0.003
	Sum of 18:1 *cis*	0.97^{a}	0.97^{a}	1.14^{c}	1.08^{c}	1.02^{b}	1.13^{c}	0.04	0.006
	18:2 *cis*-9, *cis*-12	0.96^{a}	0.95^{a}	2.13^{c}	1.64^{c}	1.15^{b}	0.77^{a}	0.26	0.001
	18:2 *cis*-9, *trans*-11	0.09^{b}	0.08^{b}	0.06^{a}	0.07^{b}	0.08^{b}	0.09^{b}	0.01	0.133
	18:3 *n*-3	1.35^{b}	1.19^{a}	2.27^{c}	1.73^{b}	1.41^{b}	1.06^{a}	0.20	<.001
	12:0	0.60^{b}	0.59^{b}	0.78^{b}	0.67^{b}	0.83^{b}	0.48^{a}	0.14	0.191
	14:0	1.55^{a}	1.59^{a}	1.78^{a}	1.74^{a}	2.06^{a}	1.81^{a}	0.38	0.791
	16:0	17.8^{a}	17.9^{a}	17.4^{a}	19.0^{a}	16.8^{a}	17.6^{a}	1.09	0.514
	18:0	51.1^{a}	50.8^{a}	44.8^{a}	49.0^{a}	43.9^{a}	52.1^{a}	6.47	0.723

8	18:1 *trans*-11	4.22[b]	4.43[b]	3.75[b]	3.51[a]	4.17[b]	4.81[b]	0.50	0.190
	Sum of 18:1 *trans*	5.22[b]	5.42[b]	4.66[b]	4.45[a]	5.24[b]	5.93[b]	0.58	0.212
	Sum of 18:1 *cis*	0.97[a]	0.97[a]	0.99[a]	0.87[a]	1.07[a]	1.05[a]	0.12	0.198
	18:2 *cis*-9, *cis*-12	0.67[a]	0.68[a]	0.72[a]	0.46[a]	0.68[a]	0.49[a]	0.12	0.198
	18:2 *cis*-9, *trans*-11	0.07[a]	0.07[a]	0.05[a]	0.05[a]	0.04[a]	0.03[a]	0.02	0.630
	18:3 *n*-3	1.01[b]	0.97[b]	0.96[b]	0.67[a]	0.84[b]	0.73[a]	0.09	0.015
24	12:0	0.38[a]	0.37[a]	0.39[a]	0.35[a]	0.35[a]	0.43[a]	0.07	0.869
	14:0	1.54[a]	1.61[a]	1.61[a]	1.50[a]	1.84[a]	1.22[a]	0.26	0.379
	16:0	16.6[a]	17.3[a]	15.9[a]	17.0[a]	21.0[a]	15.7[a]	2.25	0.254
	18:0	58.2[a]	55.0[a]	56.0[a]	55.8[a]	50.7[a]	61.0[a]	5.94	0.656
	18:1 *trans*-11	4.20[a]	5.16[a]	4.87[a]	5.15[a]	4.90[a]	3.98[a]	0.94	0.723
	Sum of 18:1 *trans*	5.21[a]	6.24[a]	6.07[a]	6.25[a]	5.75[a]	4.81[a]	1.02	0.656
	Sum of 18:1 *cis*	0.88[a]	0.90[a]	0.91[a]	0.87[a]	0.88[a]	0.84[a]	0.06	0.882
	18:2 *cis*-9, *cis*-12	0.05[b]	0.07[b]	0.05[b]	0.03[a]	0.03[a]	0.06[b]	0.01	0.022
	18:2 *cis*-9, *trans*-11	0.61[a]	0.52[a]	1.04[b]	0.53[a]	0.55[a]	0.37[a]	0.13	0.005
	18:3 *n*-3	0.78[c]	0.72[c]	0.75[c]	0.60[a]	0.58[a]	0.63[b]	0.05	0.006

A two-way ANOVA was also conducted upon raw data for linoleic acid to assess the effect of different concentrations and incubation times on fatty acid profiles (Appendix CD). SED: Standard Error of Differences of means, P: significance of effect of LA concentration and incubation time (P=<0.05), ND= Not Detectable. Values within the same row were allocated different superscripts when significantly different.



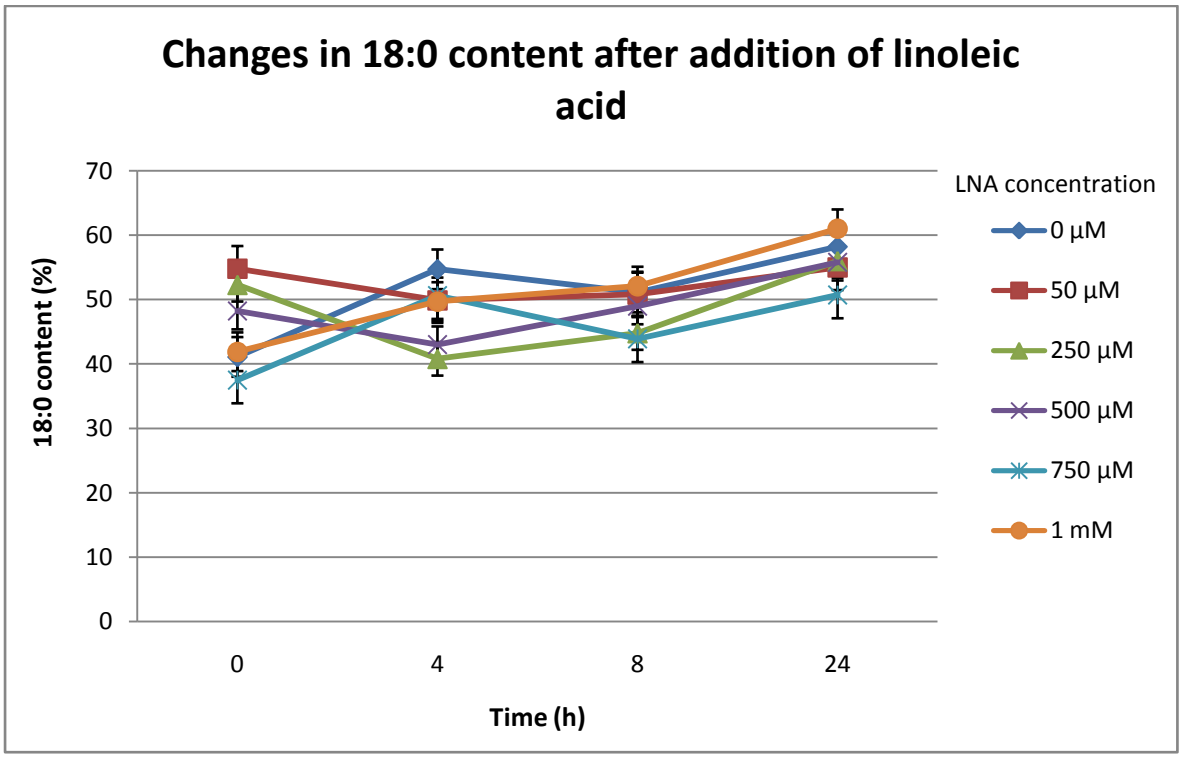
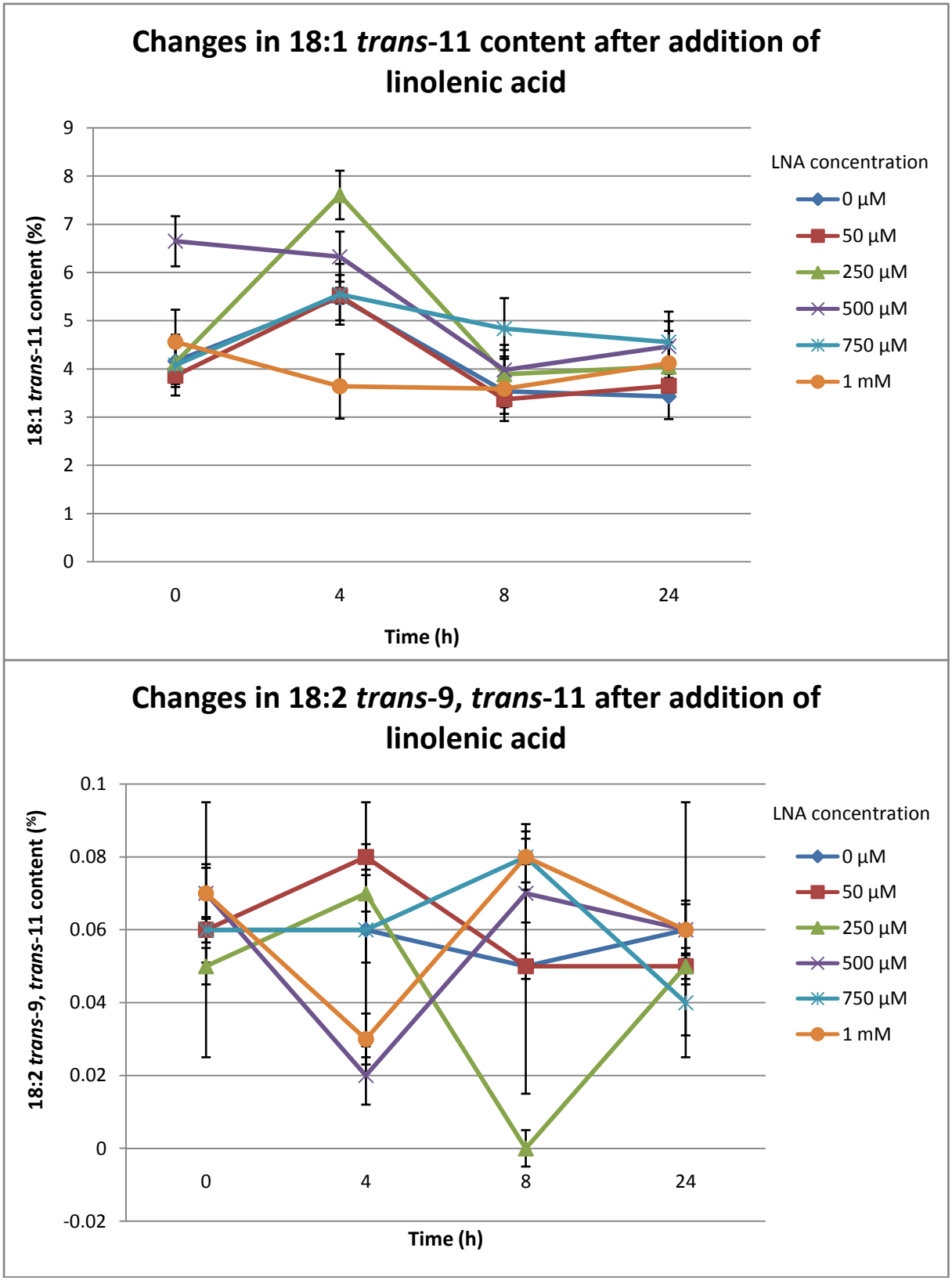


Figure 2.1: Changes in detectable 18:0 (% of total lipids) after addition of LNA and LA (µM) over the 24 hour time period. Error bars represent SED.


Changes in 18:1 trans-11 content after addition of linolenic acid
18:1 trans-11 content (%)
9
8
7
6
5
4
3
2
1
0
0
4
8
24
Time (h)
LNA concentration
0 µM
50 µM
250 µM
500 µM
750 µM
1 mM
Changes in 18:2 trans-9, trans-11 after addition of linolenic acid
18:2 trans-9, trans-11 content (%)
0.1
0.08
0.06
0.04
0.02
0
-0.02
0
4
8
24
Time (h)
LNA concentration
0 µM
50 µM
250 µM
500 µM
750 µM
1 mM

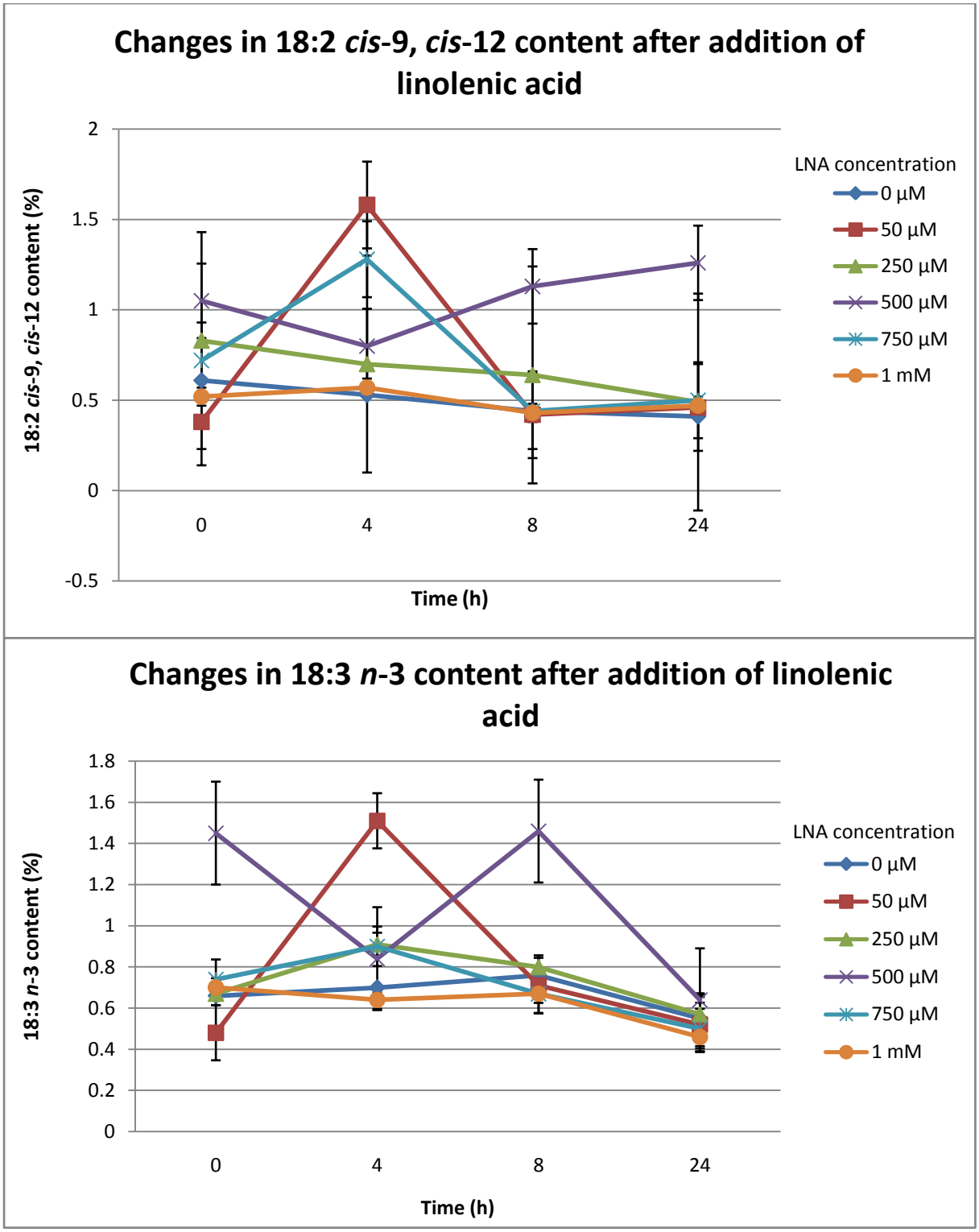
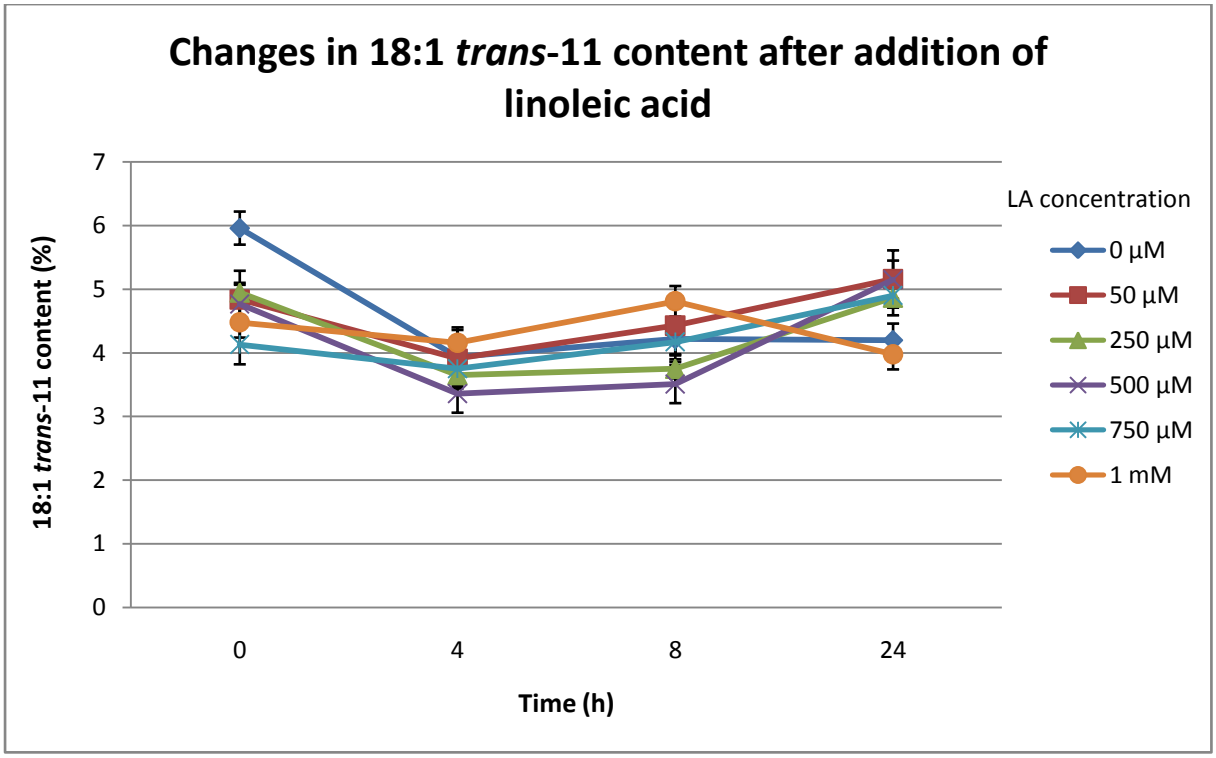
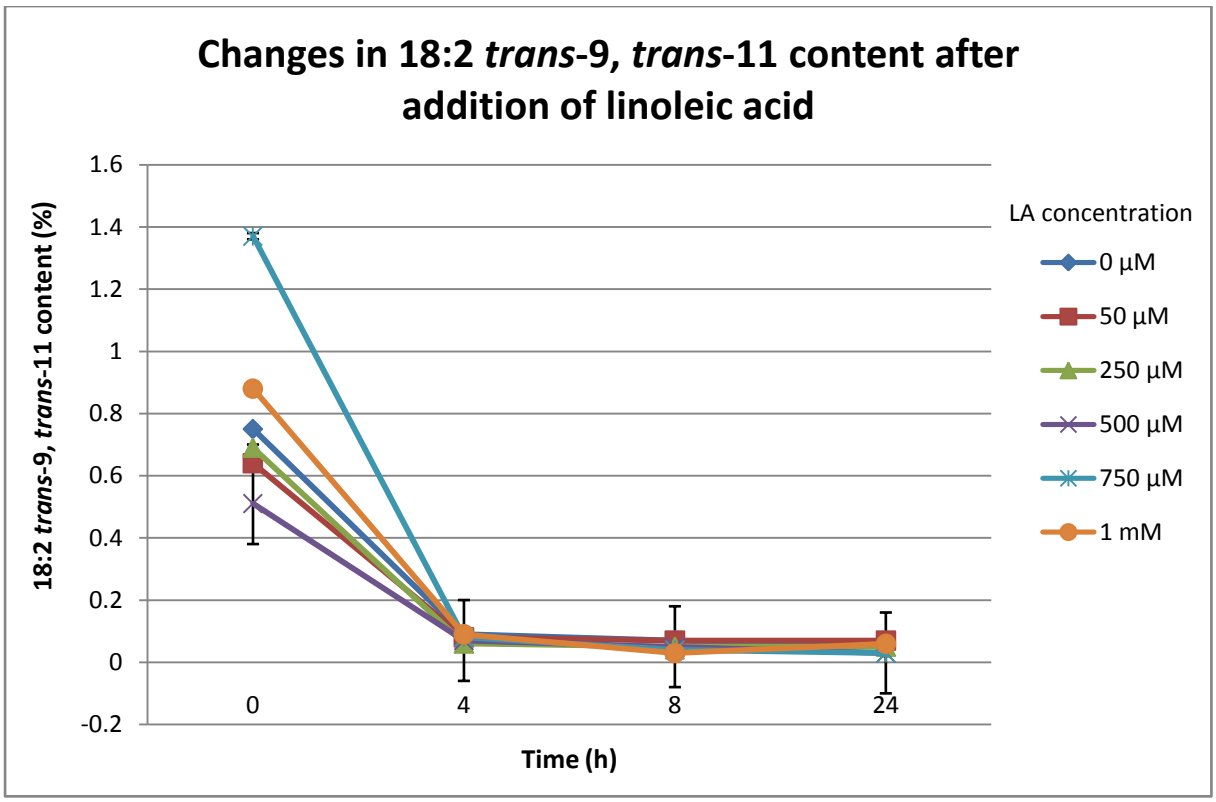
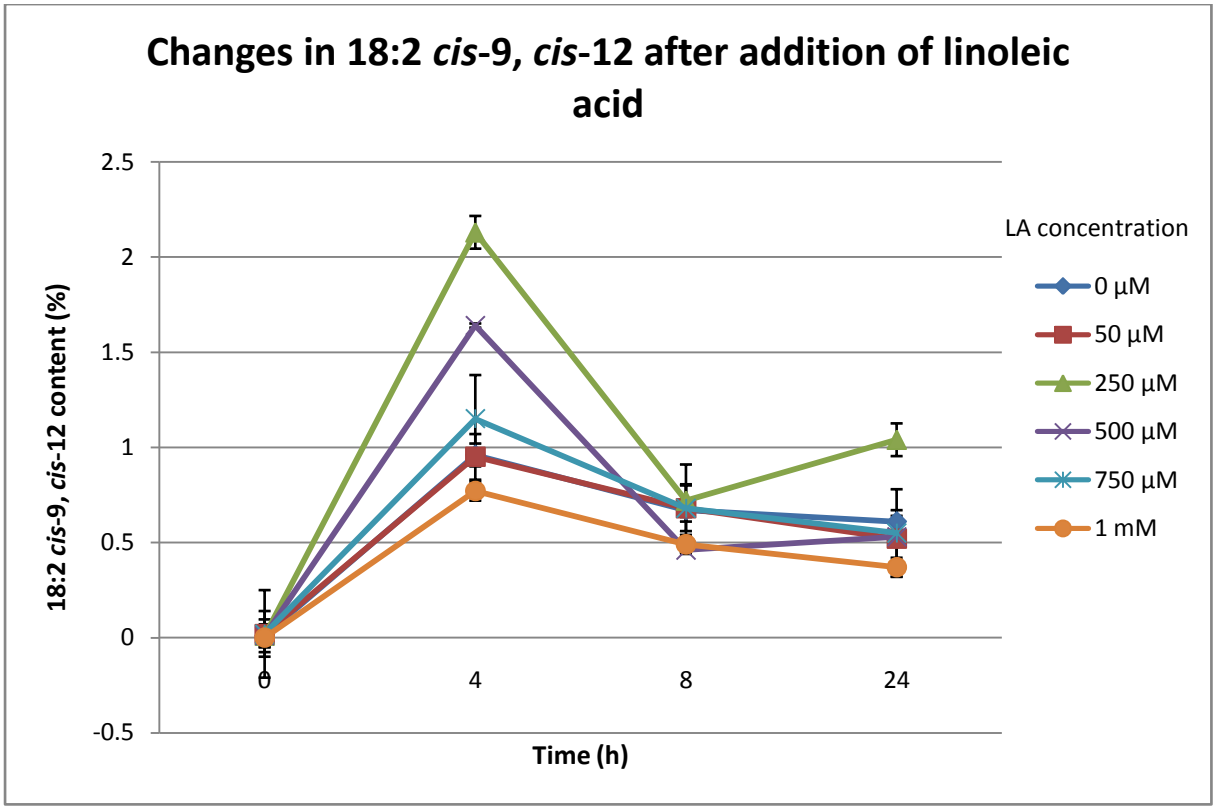


Figure 2.2: Changes in 18:1 *trans*-11, 18:2 *trans*-9, *trans*-11, 18:2 *cis*-9, *cis*-12 and 18:3 *n*-3 PUFA (% of total lipids) at 0 h, 4 h and 24 h upon addition of LNA (µM). Error bars represent SED.


Changes in 18:1 trans-11 content after addition of linoleic acid
18:1 trans-11 content (%)
7
6
5
4
3
2
1
0
0
4
8
24
Time (h)
LA concentration
0 µM
50 µM
250 µM
500 µM
750 µM
1 mM


Changes in 18:2 trans-9, trans-11 content after addition of linoleic acid
18:2 trans-9, trans-11 content (%)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
0
4
8
24
Time (h)
LA concentration
0 µM
50 µM
250 µM
500 µM
750 µM
1 mM



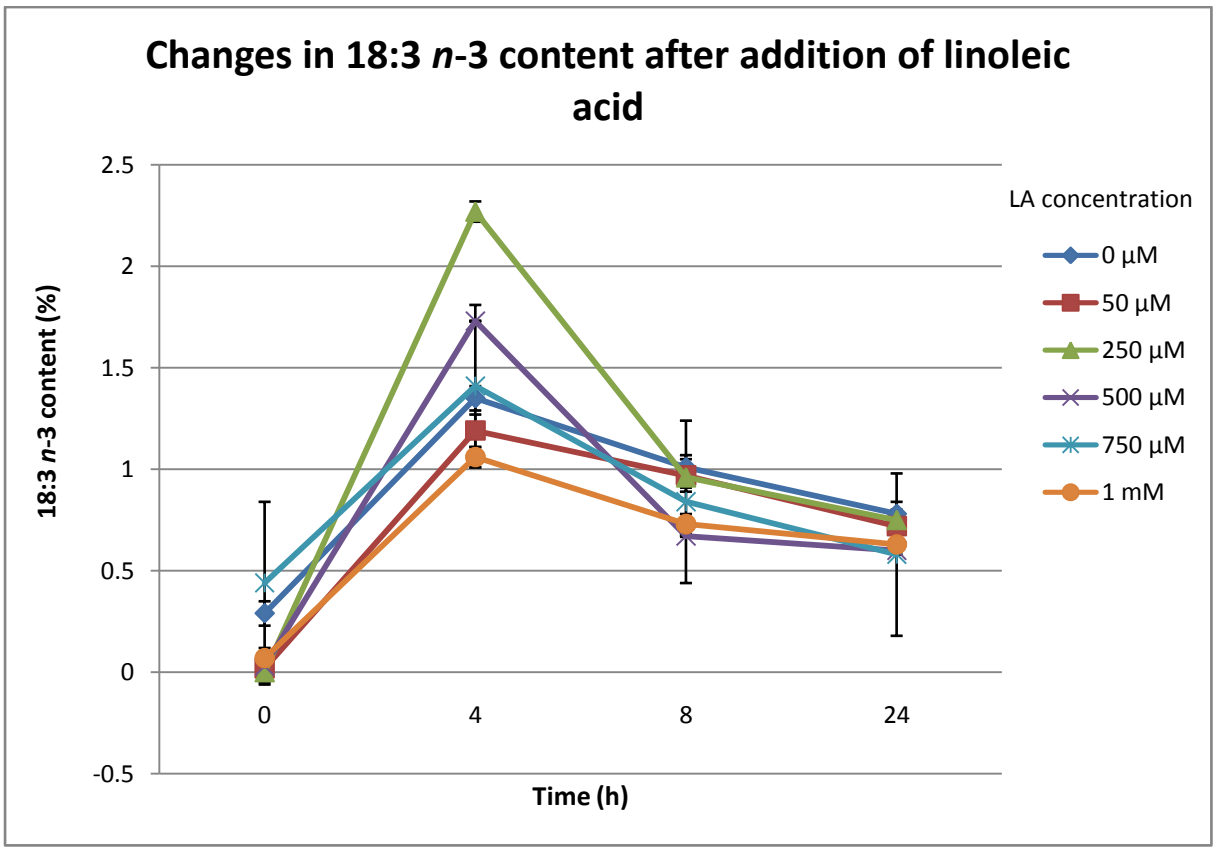


Figure 2.3: Changes in 18:1 *trans*-11, 18:2 *trans*-9, *trans*-11, 18:2 *cis*-9, *cis*-12 and 18:3 *n*-3 PUFA (% of total lipids) at 0 h, 4 h and 24 h upon addition of LA (µM). Error bars represent SED.

2.3.2: DNase treatment and Lithium Chloride precipitation

All samples were treated to minimise contamination by DNA before reverse transcription (Fig 2.4), however RNA taken from samples using LNA proved to be more problematic, therefore lithium chloride precipitation was used in conjunction with RNase-free DNase (ThermoScientific, UK) (Fig 2.4).

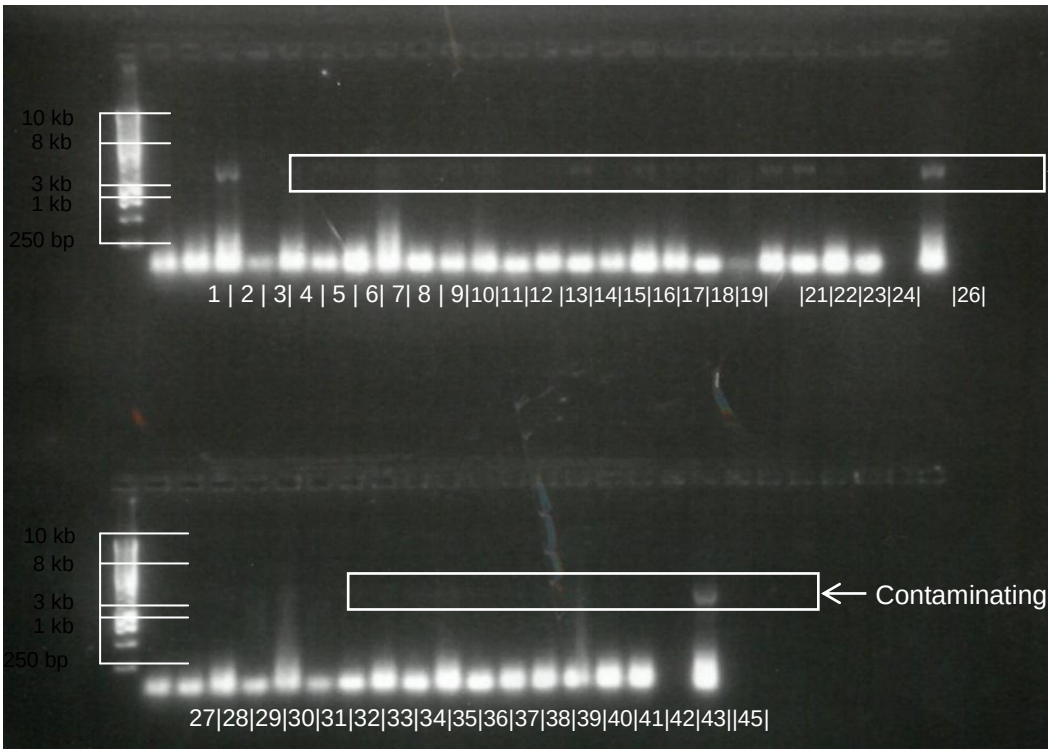


Figure 2.4: Trans UV image using QuantityOne software and Molecular Imager (Bio-Rad, UK) of PCR products from LA samples on 1% agarose gel, following RNase-free DNA treatment and electrophoresis. Lanes 2 – 10 contain controls for all time points at 0μM, 11- 24 and lane 28 contain samples from 4 h, with concentrations in ascending order and lanes 29 – 43 contains samples from 24 h with concentrations also in ascending order. First lanes contain 1kb ladder (Promega, UK) and lanes 26 and 45 (end lanes) contain blanks.

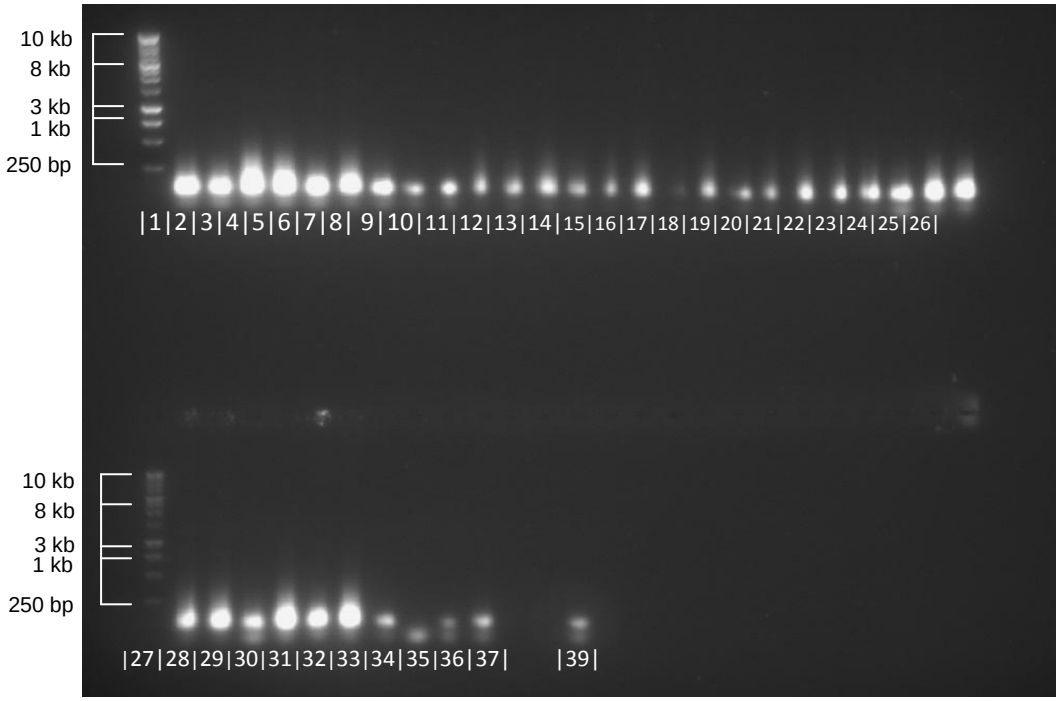


Figure 2.5: Trans UV image using QuantityOne and Molecular Imager (Bio-Rad, UK) of PCR products from LNA samples on 1% agarose gel, following RNase-free DNase and Lithium Chloride treatment and electrophoresis. Lanes 2-13 contain controls for all 4 time points at 0μM, lanes 14-25 contain 4 h with concentrations in ascending order and lanes 26 and 28-37 containing 24 h samples with concentrations in ascending order. First lanes contain 1kb ladder (Promega, UK) and lane 39 (end lane) contains a blank.

2.3.3: Reverse transcription to cDNA

After reverse transcription and PCR, the gel was visually assessed for banding at the 1300 kb point. cDNA bands around the 1300 kb level indicate amplified DNA from the microbial communities within each sample ensuring that there is a sufficient amount of genetic material to continue (Fig 2.6).

The majority of samples displayed banding at the 1300 kb level, however some did not- in lanes: 6, 18, 35, 36, 45, 65, 66, 67, 73 and 74 (Fig 2.6). These lanes equate to one control, one sample from 24h using 250 μM LNA, two samples from 24 h with 750 μM LNA and one sample from 24 h with 1mM LNA. Then two 24 h samples using 1mM LA, one 4 h sample using 250 μM LA and one control.

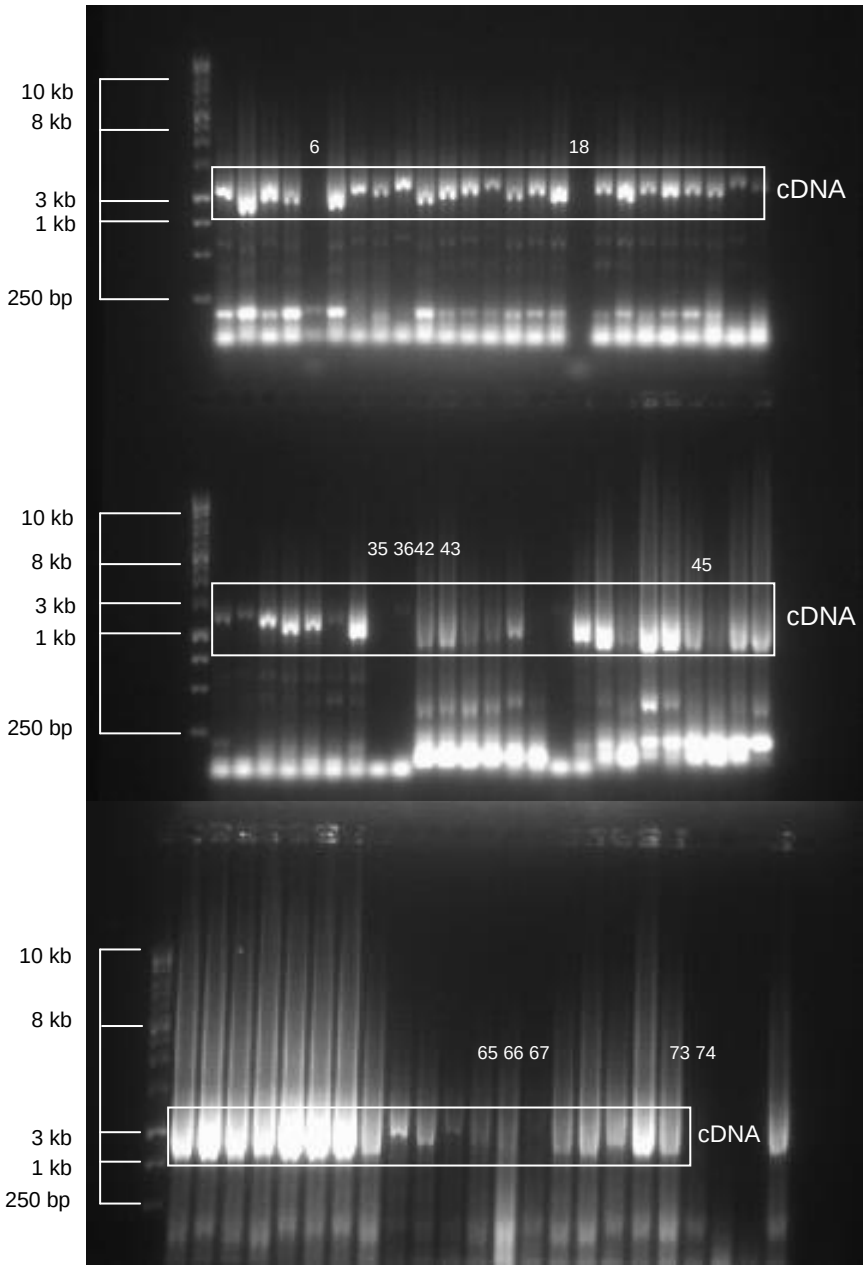


Figure 2.6: Trans UV image using QuantityOne and Molecular Imager (Bio-Rad, UK) of PCR products from cDNA from LA samples (lanes 2-33) and LNA samples (lanes 36 – 78).PCR products were run on a 1% agarose gel, following reverse transcription (SuperScript III kit, Invitrogen UK) and electrophoresis. Two empty lanes were left between LA and LNA samples at lanes 42 and 43, all first lanes contain 1kb ladder (Promega, UK) and last lane in third image shows an empty lane followed by a blank with some contamination.

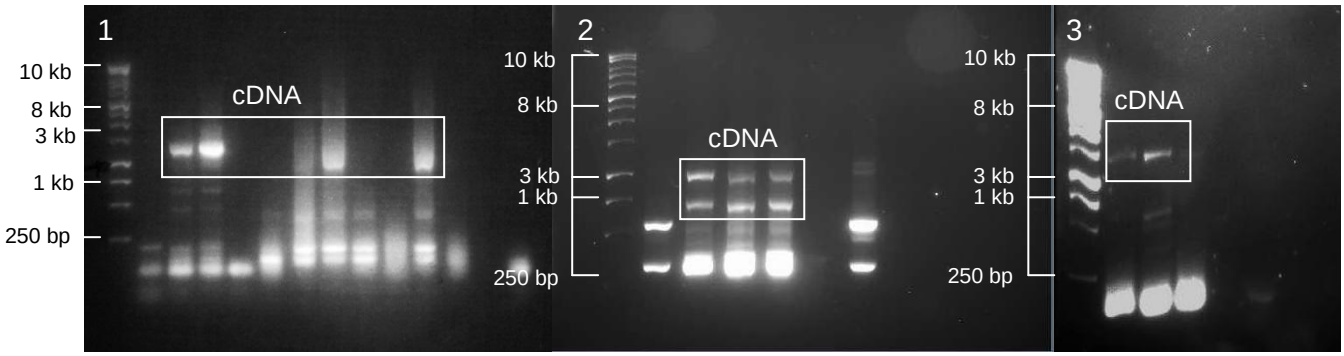


Figure 2.7: Trans UV image using QuantityOne and Molecular Imager (Bio-Rad, UK) of PCR products that did not run previously. Image 1 shows successful banding at the 1300 kb level in lanes 3, 4, 7, 8 and 11. Image 2 show 5 previously unsuccessful samples with lanes 3, 4 and 5 showing the appropriate fluorescence. Image 3 shows successful banding of 3 previously unsuccessful samples

Unsuccessful samples were first run again on a 1% agar gel, resulting in five showing the expected banding (Image 1, Fig 2.7). Then, using fresh cDNA for samples 5, 6, 35, 60, 70 and 71 the appropriate fluorescence was displayed in 6, 35 and 60 (Image 2, Fig 2.7) and when using a higher concentration in the PCR banding at the 1300 kb level for samples 5, 70 and 71 was evident.

2.3.4: Terminal restriction fragment length polymorphism (TRFLP)

Unweighted, pair group methoddendograms revealed, over all there was no significant clustering caused by time or LA/LNA concentration (Fig 2.8 and Fig 2.9). There is no relationship between inhibition of biohydrogenation (mostly in 250 µM and 500 µM samples) and a shift in bacterial diversity of the rumen using this technique of bacterial diversity profiling. Although some weak clustering patterns may be observed with 1mM LA samples, it does not indicate a major change in the rumen bacterial population (Appendix CD).

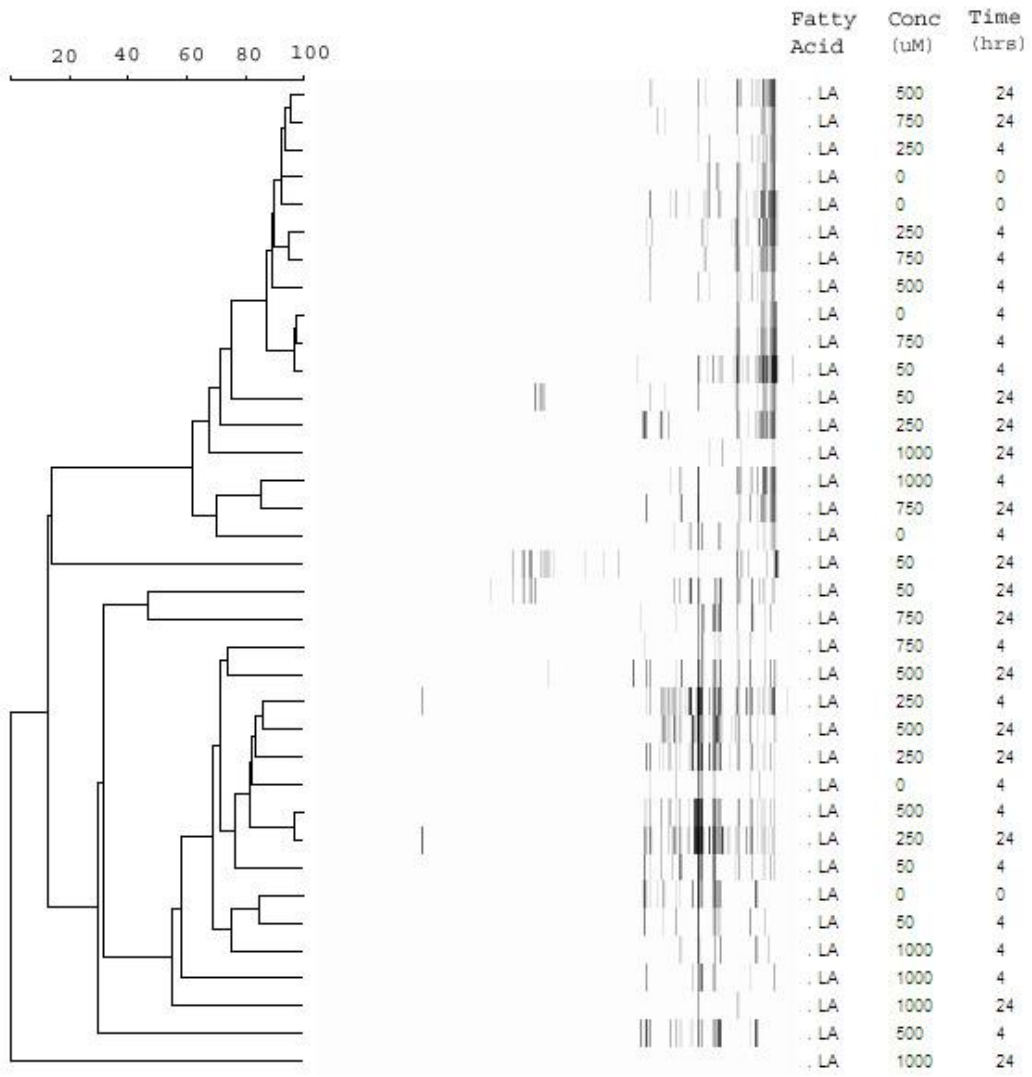


Fig 2.8: Unweighted group pair method with dendogram showing the effects of time and LA concentration of the bacterial community of the rumen following HaeIII-based TRFLP. Scale relates to percentage similarity.

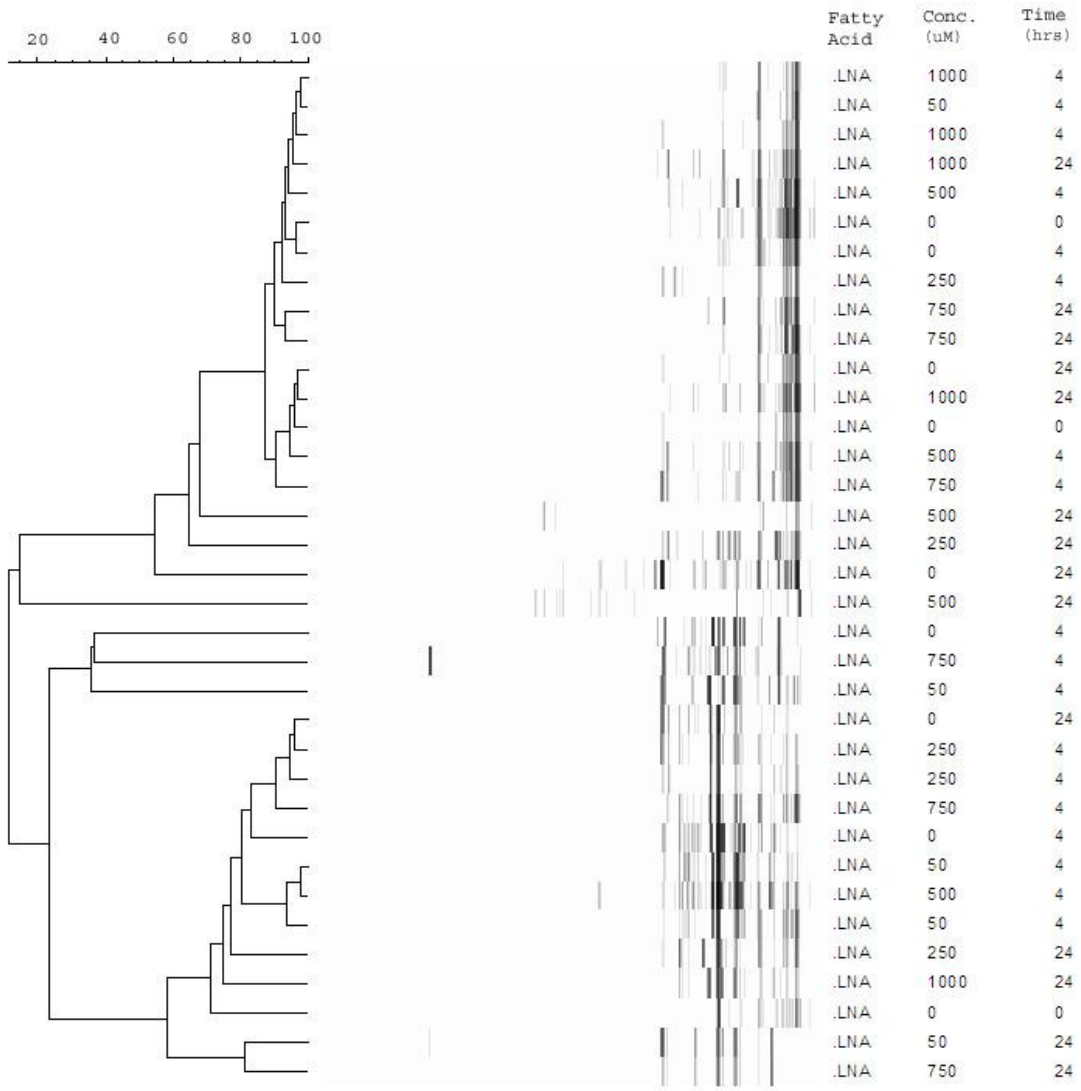


Fig 2.9: Unweighted group pair method with dendogram showing the effects of time and LNA concentration of the bacterial community of the rumen following HaeIII-based TRFLP. Scale relates to percentage similarity.

2.4: Discussion

As previously mentioned, there is limited research detailing interactions between the rumen as a whole and levels of PUFAs required to inhibit biohydrogenation. Based on the hypothesis provided by Maia *et al.*(2010) this study explored the effect of up to 1mM concentrations of LA and LNA upon microbial populations in fresh rumen fluid. The use of fresh rumen fluid ensures a more accurate simulation of the rumen and allows observation of the effects of PUFA on the whole rumen fluid-associated microbiota. On the whole, biohydrogenation seemed to be successfully inhibited by mid-level (250-500 µM) concentrations of PUFA, whilst higher concentrations did not evoke any significant response.

The observed changes in PUFA and SFA content suggest that biohydrogenation was inhibited to an extent. Although, it is somewhat surprising that there was not a more linear relationship between LA/LNA concentration and PUFA/SFA content of samples, in that the more PUFA present the less biohydrogenation occurred leading to lower levels of 18:0. Due to the very unusual relationship between LA/LNA concentration and inhibition, the issue warrants further investigation in the future. In reality, the optimum concentrations for inhibition appear to be those of a medium level, at 250 and 500 µM. Whilst the fatty acid profile of samples treated with 1mM LNA were not significantly different, there was still a reduction in 18:0 (reductions of 7% at 4 h and 5.5 % at 24 h with an increase of 0.4% at 8 h); however not as significant a difference compared to samples treated with 250 µM (a reduction of 14.8% after 4 h, 14.1% at 8 h and an increase of 2.9% after 24 h). Total 18:1 *trans*and 18:1 *cis* values for LNA show a clear quadratic relationship, and peak at 250 µM at 4 h and then at 8h and 24 h 18:1 PUFA content increases with LNA concentration. In contrast, total 18:1 *cis* and 18:1 *trans* values for LA do not show any such pattern, and also show a significant increase in 18:1 PUFA from controls, to treated samples (for example 18:1 *trans* jumps from 0.47% at 0 h to 5.12% at 4 h and then 5.93 at 8 h; total 18:1 *cis* increases from 0.24 at 0 h to 4.97% at 4 h, 5.22% at 8 h and 5.21% at 24 h). Samples treated 1mM LA showed mixed reactions: 18:0 content decreased by 5% after 4 h, then at 8 h increased by 1% and by 2.8% after 24 h. Although in keeping with the pattern observed in LNA addition, the most significant reduction was achieved upon addition of 250 µM LA (with reductions of 13.6% after 4 h, 6.3% after 8 hand of 2.2% after 24 h). The sum of 18:1 *trans* in terms of LA shows a clear increase over time and total 18:1 *cis* peaks at 4 hours (from 0.08% at 0 h, to 1.14 at 4 h and 0.99% at 8 h), demonstrating a clear increase in 18:1 PUFAs over time. However, the sum 18:1 *trans*and 18:1 *cis* for LNA show less clear changes over time, although a weak linear relationship between time and increased 18:1 PUFAs content can be observed. In terms of evaluating the efficacy of LA compared to that of LNA, it is not as clear cut as previous experiments would suggest, as both fatty acids have

had roughly the same effect, however when looking in more detail it would appear LNA elicited a slightly more pronounced effect in lowering the 18:0 content and LA was more effective in increasing PUFA (18:2 *n*-6 and 18:3 *n*-3) content in this instance.

The majority of variation can perhaps be explained by the use of a mixed culture in fresh rumen fluid. Despite the rumen fluid being thoroughly mixed, there will always be subtle differences in population between each inoculum which will lead to a difference in fatty acid composition.

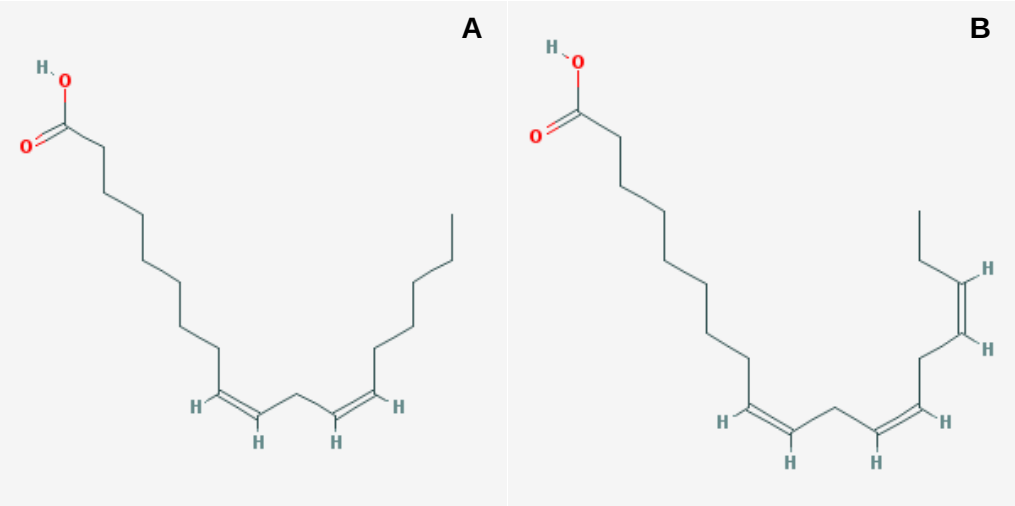


Figure 2.10: Molecular structure of linoleic (A) and α-linolenic acid (B) from PubChem, NCBI.

This is contrary to the expected, when considering the hypothesis that PUFA disturbs the lipid bilayers in rumen microbes. LA and LNA have very similar structures, with LNA having one extra double bond, and therefore another kink in the chain (Fig 2.10). As such, it may be expected that LNA would inhibit biohydrogenation more efficiently and would cause disruption in the bacterial community. This hypothesis was tested by Maia *et al* (2007), who were able to rank four different PUFA in terms of their toxicity to the growth of rumen bacteria: Eicosapentaenoic acid (EPA)>Docosahexaenoic acid (DHA)>LNA>LA. However in this case, when using LNA there were no changes in the microbial population and less significant differences in 18:0 and PUFA content when compared to samples treated with LA.This may be unrelated to the number of double bonds in the fatty acid, but the position of the double bonds, perhaps the structure of LA provides a larger disruption of the bacterial cell membrane, causing more damage. Further research is required into why PUFA is so toxic to rumen bacteria, and the factors which make an unsaturated fat toxic.

The effect of time upon the fatty acid profiles of samples was also significant, with the most notable reductions in 18:0 occurring at 4 and 8 h, and most significant changes diminishing by 24 h. At 4 h, there was a reduction of 14.8% in 18:0 with 250 µM LNA and a difference of 13.9% when using 250 µM LA. However, after 24 h, the samples showed an increase of 2.9% in 18:0 with LNA, but with LA the 18:0 content decreased by 2.2% respectively, compared to controls. By 24 h polyunsaturated fats (18:2 *n*-6 and 18:3 *n*-3) had also decreased to levels consistent with controls. When using 250 µM LA, at 4 h the concentration of 18:3 *n*-3 was 2.27% and 18:2 *n*-6 content 2.13%, however by 24 h, concentrations had dropped to 0.75% and 1.04% respectively. Whilst this level of PUFA is higher than those in controls, it is still not as high as levels achieved at 4-8 hours. When treat with 250 µM LNA samples began with 0.83% of 18:2 *n*-6 and 0.67% of 18:3 *n*-6, this increased by 0.02% and 0.24% respectively. Then after 24 h, 18:2 *n*-6 content declined to 0.05% as in controls, and 18:3 *n*-3 decreased further to 0.57%. Across the sampling times, there is a clear peak in the reduction of 18:0 and in the elevation of 18:2 *n*-6 and 18:3 *n*-3, with the effects declining as time progresses. This suggests that bacterial populations began to recover relatively quickly, around 24 h after exposure to high concentrations of PUFA. Rumen bacteria have shown adaptation to several compounds, including essential oils and plant extracts with some studies describing resistance to Nisin and hydrolysable and condensed tannins (Cardozo *et al.*, 2004; Molero*et al.*, 2004; McSweeney*et al.*, 2001; Russell &Mantovani, 2002). In some cases this has led to erroneous conclusions with relation to the true effects of compounds on ruminal activity (Calsamiglia*et al.*, 2007). Thus, it is entirely feasible that between 8 and 24 hours, bacteria have simply recovered and managed to remove enough double bonds to continue biohydrogenating PUFA at a 'normal' level (i.e.: comparable to levels found in controls).

It is difficult to compare this data to previous experiments, as most studies have used only pure cultures and have not explored the temporal effects upon the final fatty acid profile. The sensitivity observed in pure culture experiments (for example from Maia *et al.*, 2010 and 2007) was not reproduced here possibly due to the mixed culture allowing the rumen microbiome to function as a whole, not just selected bacterial species. This could mean that PUFA was metabolised quicker in comparison to when individual species of bacteria are used. It may also be interesting to repeat this experiment using 10% rumen inoculum, as this would give the mixed microbial population more space to fluctuate and could possibly make results more clear. At 50% rumen fluid, there is also the confounding factor of high C18:0 carry over from the rumen.

Overall, LA/LNA addition caused no change in branched and odd chain PUFA which can be interpreted as a crude indicator that there were few changes in the microbial

community during the experiment (Appendix CD). This is reassuring, as there is potential for the ruminal microbiome to become compromised, interfering with the animal's digestion.

Having confirmed that biohydrogenation can be inhibited by addition of PUFA, the next step is to consider methods for achieving such levels. Both linoleic and linolenic acid are abundant in forage, and the typical PUFA content of fresh grass is well researched, with approximately 5.03 g/kg of 18:2 *n*-6 and 17.4 g/kg of 18:3 *n*-3 (Huws *et al.*, 2009). Theoretically, this amount of LA and LNA in forage should be enough to inhibit biohydrogenation in the rumen, without the need for selectively bred, high PUFA forage:

The capacity of the rumen varies greatly depending on the size of the animal and general estimates can vary between 110 to 235 litres (here we assume that the average capacity is 150 L), 12% of which is dry matter (DM), which converts to approximately18 kg (Frandson, Wilke & Fails, 2003).

LNA molecular weight = 278.435
1µM LNA = 0.000278435 g/L
250µM LNA = 0.0696 g/kg
500µM LNA =0.1312 g/kg

LA molecular weight = 280.445
1µM = 0.000280445 g/L
250µM = 0.0701 g/kg
500µM = 0.1402 g/kg

Therefore, 0.069 g/L LNA and 0.131 g/L LA would theoretically be present in the rumen when treated with 250 µM and 500 µM LNA and using the same concentrations of LA, 0.070 g/L and 0.140 g/L respectively.

Already available through a typical forage diet are 1.944 g/L of LNA and 0.604 g/L of LA, and, assuming an average ruminal volume of 150 L, 291.6 g/150L and 90.5 g/150L respectively (Huws *et al.*, 2009):

LNA: 18 × 17.4 = 291.6/150 = 1.944 g/L
LA: 18 × 5.03 = 90.5/150 = 0.60 g/L
(18 kg total DM in the rumen. 17.4 g/kg DM of LNA and 5.03 g/kg DM or LA based upon the fatty acid composition of fresh forage as reported by Huws et al., (2009).

As such, it is clear that there is enough LA and LNA present in fresh grass to potentially inhibit biohydrogenation, however due to the drip feeding nature of ruminant consumption, the full amount is never present in one lump sum, it is instead, slowly fed into the bovine digestive system over time.

In addition, bacteria naturally form biofilms on both feed particles and on the rumen wall for protection, however *in vitro* there is no opportunity for the formation of biofilms, or any suitable surfaces for adherence (McAllister *et al.*, 1994). Therefore, it is possible that microorganisms could be more susceptible *in vitro* in comparison to in the rumen, and that a higher concentration of PUFA may be necessary *in vivo*.

Nonetheless, if lipase activity could be enhanced, then more PUFA would be available in the rumen, which could inhibit biohydrogenation. The enhancement of lipolysis and its effects subsequently on biohydrogenation is tested within the next experimental chapter.

3: Enhancing activity of rumen lipases to inhibit biohydrogenation *in vitro*.

3.1: Introduction

Lipolysis and associated lipases were first identified in the rumen by Garton*et al.* (1958) and were generally thought active in the ruminant's oral cavity and saliva. However, Garton

(1961) later found that there was no lipase activity in sheep saliva or in the defaunated rumen, and thus concluded that the microorganisms of a healthy rumen were responsible for lipolysis. Subsequently, fractionation of rumen contents by differential centrifugation has revealed that rumen bacteria are mostly responsible for lipolytic activity. Despite this, research continues to be directed into the activity of plant and salivary lipases (Faruque*et al.*, 1974). Whilst subsequent studies have shown the activity of such lipases to be much lower in concentrate-fed animals, forage is rich in galacto- and phospholipases which remain active in the rumen for up to 5 h (Faruque*et al.*, 1974, Lee *et al.*, 2004). As such, it is feasible that in grazing ruminants, plant lipases do contribute to lipolysis.Nonetheless, such contribution is thought to be minimal, and the majority of hydrolysis is carried out by microbial lipases, more specifically, those from bacteria and fungi (Lourenco *et al.*, 2010; Dawson *et al.*, 1977). Lipases play an essential role in rumen lipid metabolism as the providers of non-esterified free fatty acids which form the substrates for biohydrogenation. Based on the hypothesis that PUFAs are probably toxic to biohydrogenating rumen bacteria, enhancing lipolysis and subsequent release of free fatty acids may be an effective means of inhibiting biohydrogenation.

When considering the types of lipase in the rumen, it is important to take into account the diet of the animal, for example: a concentrate-based diet may engage higher activity from *Anaerovibriolipolytica* lipases in order to process triglycerides; whereas a forage-based diet would require more input from phospholipases and galactolipases to hydrolyse phospholipids and glycolipids (Henderson, 1971).

Recently, data mining of metagenomes has provided valuable new insights into microbial ecosystems and their potentially novel metabolites, which would otherwise have remained undiscovered. Metagenomics provides a culture-independent alternative for assessment and exploitation of complex microbiomes, such as the rumen, and allows access to the whole microbial community. The Institute of Biological, Environmental and Rural Sciences at Aberystwyth University, is home to an extensive metagenomic library of rumen microbes, using phage-based and fosmid-based cloning systems. From this library, 14 lipolytic and 2 phospholipase genes were described, expressed and characterised (Privé*et al.*, 2011; 2013; 2015). Six lipases were classified as members of family VII, five were characterised as new members of the lipase subfamily I, two lipases were included in family IV and the last was classified in the esterase family II (GDSL). The two putative phospholipases, Pl1 and Pl2ss were identified as outer membrane proteins, and Pl2ss predicted to have a patatin-like domain (often observed as virulence factors in pathogenic bacteria). Pl1 showed a preference towards short to medium chain length triglycerides and optimum operating conditions were at 30°C and pH 7-7.5. Pl2ss showed no substrate

specificity and favoured higher temperatures around 45°C and a more alkaline environment at pH 8.5 (Privé, 2011). Whilst these enzymes operate slightly more efficiently under different parameters to those of the rumen, the pH and temperature ranges remain relatively similar to the 38-42°C and pH 6-7 seen in the rumen.

In this study an enhancement of lipolysis and subsequent effects on lipolysis was investigated using the two phospholipases (Pl1 and Pl2ss) characterised by Privé(2011), along with a commercially available lipase from fungi*Thermomyceslanuginosus*(A1; Sigma Aldrich, UK). *Thermomyceslanuginosis*is a widely distributed thermophilic fungus, mostly isolated from self-heating masses of organic debris its numerous enzymes are therefore more heatstable in comparison to those from other mesophiles (Singh *et al.*, 2003). The A1 enzyme is a thermostable lipase which has found applications in many different areas, from biodiesel to fine chemicals (Fernandez-Lafuente, 2010). It is a single chain protein of 269 amino acids and a molecular mass of 31 KDa with a strong tendency to form bimolecular aggregates (Sigma Aldrich, 2014). A1 readily hydrolyses lipids to form free fatty acids, and is specific for positions 1 and 3 (Fernandez-Lafuente, 2010).

A study by Huxley (2012) using Pl1, Pl2ss and A1 showed significant increases in 18:3*n*-3 and 18:2*n*-6 content during incubations, and consequently increased lipolysis. Whilst levels of PUFA required to inhibit biohydrogenation were not fully achieved, it is quite possible that the use of higher concentrations of Pl1, A1 and Pl2ss, and possibly longer incubation times could allow higher levels of PUFA release to be achieved.

The following experiment builds upon Huxley's work as a basis to build upon, using similar substrates, incubations and sample times and the same phospholipases at higher concentrations (75, 100, 125 and 150 µM).

3.2: Materials and Methods

3.2.1: Chloroform: methanol extraction procedure

Fresh perennial ryegrass (PRG, *Loliumperenne,* AberDart) was freeze-dried and ground using an electrical mill, and stored at -20°C prior to lipid extraction. Using a four point digital balance, 25g of freeze dried PRG was weighed into a polytetrafluoroethylene (PTFE)-lined screw top conical flask. In the fume cupboard, 125 mL of chloroform: Methanol ($CHCl_3$: MeOH; 2:1) and 100 µL of internal standard (C23:0, 15 mg/mL $CHCl_3$) was added. The flask was secured with a PTFE-lined cap, gently swirled left and right, and then allowed to stand for 20 min.

Using a vacuum flask with a funnel lined with muslin and an electronic pump, the contents of the flask was emptied slowly into the funnel. The solvent was filtered out of the dry matter by suction from the pump and collected in the clean flask. In order to maximise the lipid extracted from the dry matter, the process was repeated using an additional 100 mL and then 80 mL of chloroform: methanol – each being allowed to stand for 20 min. The pump was used until the liquid matter being drawn out ceased to flow, or slowed to a negatable amount.

Once extracted, 6 mL of the liquid extract was dispensed into 21 pre-labelled extraction tubes, which were then sealed with PTFE-lined caps and stored at -20°C.

3.2.2.1: Lipid preparation post-extraction

Two days prior to the scheduled experiment, the extraction tubes containing the chloroform: methanol and phospholipid extract were placed in the freeze drier for 48 h. All liquid was removed, leaving a film-like residue containing the phospholipids. Nitrogen was added to each of the tubes headspace and then sealed using PTFE lined caps.

3.2.2: Purification of the phospholipases Pl1 and Pl2ss

Escherichia coli (genotype TOP10) cells containing the phospholipase genes were streaked onto 2 LB nutrient agar plates containing 100 µM ampicillin (Sigma-Aldrich, UK). The plates were incubated at 37°C overnight, then a single colony was selected, and inoculated into 10 mL LB nutrient broth containing filter sterilised 100µM Ampicillin. These cultures were incubated at 37°C and shaken at 200 rpm overnight. The starter culture was then transferred into 50 mL of fresh LB medium containing 100µM ampicillin (1% inoculum).

These were incubated at 37°C for 3 h and shaken at a rate of 200 rpm until the OD reached 0.6. During incubation, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was prepared, using 2.38 g in 10 mL of distilled water which was then filter-sterilised. Samples (two eppendorfs of 2 mL) were taken from the cultures for the un-induced state (0 h) before 500 µL of IPTG was added to each flask to induce protein expression.

Both cultures were then incubated at 37°C and shaken at 200 rpm for 4 h, with a 2 mL sample taken every hour, to monitor OD and to perform SDS PAGE.

After 4 h, the culture was separated into the appropriate number of 50 mL centrifuge tubes, and spun at 10,000 rpm for ten minutes at 4°C. The supernatant was discarded and the pellets containing cells stored at -80°C overnight in preparation for protein purification.

3.2.3: Enzyme purification

Purification of the proteins was carried out in native conditions using the Ni-NTA (Nickel - Nitrilotriacetic Acid) agarose affinity chromatography system (Quiagen, UK). The cell pellet prepared in chapter 3.2.5.1 was re-suspended in 20 mL native binding buffer at pH 8.0 (15 mM Imidazole, 500 mM NaCl, 20 mM TrisHCl, see Table 3.1) and 500 μL of lysozyme (10mg/100mL) added. Then, 2 protease inhibitor tablets were dissolved per 20 mL of sample with thorough mixing then incubated on ice for 30 minutes.
The solution was sonicated on ice (Soniprep 150, MSE UK Ltd., UK) with six 30 sec bursts at high intensity with a 30 second cooling period between each burst. The resulting lysate was then centrifuged at 12,000 rpm for 20 minutes at 4°C and the supernatant transferred to a clean tube.

Table 3.1: Composition of Native binding buffer

Component	For 200 mL	For 400 mL
15 mM Imidazole	204 mg	408 mg
500 mM NaCl	5.84 g	11.68 g
20 mM TrisHCl	480 mg	960 mg

Purification was then approached using two different methods: Affinity chromatography and the Ni-NTA Agarose kit (Qiagen, UK).

The first method employed,used the Ni-NTA Agarose kit following manufacturer's guidelines.

The second method used the remaining lysate and the Ni-NTA resin from the previous kit. The resin was washed using binding buffer and 10 mL of lysate was added to the resin and incubated for 1 h at room temperature.

The solution was put in 5 mL disposable polypropylene columns (Qiagen, UK) 5 mL at a time and allowed to flow through into collection tubes. This process was repeated twice to ensure all protein was captured. Then 7.5 mL of wash buffer (Table 3.2) was allowed to drain through the column, once again the process was repeated twice to ensure thorough washing.

Then, 1 mL of elution buffer (Table 3.3) was added and allowed to drain through into a 2 mL Eppendorf tube (this process was repeated 3 times to achieve a total of 3 mL of each protein).

Table 3.2: Composition of wash buffer

Component	For 200 mL
35 mM Imidazole	476 mg
1 M NaCl	11.68 g
20 mM TrisHCl	480 mg

Table 3.3: Composition of elution buffer

Component	For 200 mL
400 mM Imidazole	5.44g
500 M NaCl	5.84 g
20 mM TrisHCl	480 mg

3.2.4: Determination of protein concentration

Protein concentration was ascertained after both purification methods using the Bradford assay (Bradford, 1976) and the Epoch Microplate Spectrophotometer (BioTek, UK). For the Bradford assay, cuvettes were filled with 1 mL Bradford reagent (Sigma-Aldrich, UK) and 33.3 μL of protein extract and run at 595 nm on Ultraspec 400 visible spectrophotometer (GE Healthcare Life Sciences, UK) using SWIFT II software.

Using the Microplate spectrophotometer, 1 μL of each protein was placed as a microdot onto the Take3 Micro-volume plate and read using Gen5 data analysis software at 280nm.

3.2.5: 2D gel electrophoresis

Using the purified samples, as described above, polyacrylamide gel electrophoresis (SDS PAGE) was carried out to visualise the purity of the end product.
Samples (15 μL) were added to 5 μL of loading buffer and heated to 95°C for 2 minutes to denature the proteins.
A 12.5% separating gel was poured into a gel caster and allowed to set (Table 3.4), then a 4% stacking gel was added and a comb inserted to create wells (Table 3.5).
Tetramethylethylenediamine (TEMED) was added just before pouring.

Table 3.4: Composition of 12.5% separating gel.

Component	For 1 mini gel
30% Acrylamide	4.2 mL
0.8% bisH_2O	3.3 mL
1.5 M Tris (pH 8.8)	2.5 mL
TEMED	5 µL
10% Ammonium Persulphate	50 µL

Table 3.5: Composition of 4% stacking gel

Component	For 2 mini gels
30% Acrylamide	1.3 mL
0.8% bisH_2O	6.1 mL
0.5 M Tris (pH 6.8)	2.5 mL
10% Sodium dodecyl sulphate (SDS)	100 µL
TEMED	10 µL
10% Ammonium Persulphate	50 µL

Samples were added to the appropriate wells, along with 10 µL of 250 kDaPrecision Plus Protein Prestained standard (Bio-Rad, UK) and then run on a BioRadPowerPac 300 at 120V for 1 h. Tris buffer saline was used to run the gels: Glycine, tris base and SDS. The gels were then removed and the protein bands were detected using Coomassie Brilliant Blue stain with gentle agitation for 2 h. The gels were rinsed gently with distilled water and background staining was removed overnight using a de-stain solution of: 10% Acetic acid, 30% Methanol and 60% distilled water.

Gels were then imaged using GS-800 Calibrated Densitometer (BioRad, UK) and QuantityOne 4.6.3.

3.2.6: Concentration of proteins

Proteins were then concentrated using the Amicon Pro Purification system (EMD Millipore, UK) following manufacturer's guidelines and quantified again using the Epoch Microplate Spectrophotometer.

3.2.7: Lipase activity

The activity of phospholipase 1 (Pl1) and phospholipase 2 (Pl2ss) was determined using ρ-nitrphenyl laurate (C12) (Sigma-Aldrich, UK). The assay consisted of 770 µL of 50 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5) (MES), 4µL of 100 mM ρ-nitrophenyl laurate in 1% Ethyl acetate and 200 µL of purified enzyme; the assay was then allowed to stand for 10 mins. Blank reading vials contained 50 mM MES, 4 µL of 100 mM laurate in 1% Ethyl acetate and 200 µL of water.

Activity was assessed both quantitatively using a spectrophotometer.

Activity was tested in triplicate based upon the amount of ρ-nitrophenol being released and was measured at 410 nm using the Ultraspec 400 visible spectrophotometer (GE Healthcare Life Sciences, UK) and SWIFT II software.

Standard curve was created using 0.05 to 0.5 µmol PNP/mL standard solution (in 50 mM MES, pH 6.5) diluted to 5 mL using MES. 1 mL of each standard was measured using 50mM MES as a blank at 410 nm in triplicate, with the dilutions moving in ascending order. The standard curve of 0.05 to 0.5 µmol ρ-nitrophenol/mL was established by plotting A_{410} readings against the ρ-nitrophenol concentration (Appendix CD).

Results from the plot were then inputted into the following equation to translate absorbance to mM of substrate hydrolysed:

$$\text{µmol ρ-nitrophenol/mL reaction mixture} = \frac{(A_{410}\text{-Y intercept})}{(\text{slope} \times 1 \text{ mL reaction mixture}}$$

Specific activity (which equates to U/mg protein) was then calculated using the following equation:

$$\text{Specific activity} = \frac{\text{µmol ρ-nitrophenol/mL}}{(\text{mg protein added} \times 10)}$$

N.B: Activity for Pl1 and Pl2ss was very low, and the volume of protein after concentration was extremely low also, so due to time constraints it was decided to proceed using several concentrations of the A1 (*Thermomyceslanuginosus*) enzyme only.

3.2.8: *In vitro* experiment

Three non-lactating, rumen-cannulated Holstein-Friesian dairy cows each provided 0.5 L of hand squeezed and strained rumen fluid. Cows were located at Aberystwyth University's

Trawsgoed Experimental Farm (Aberystwyth, Wales) and allowed free access to grazing, and to water. Sampling was carried out at approximately 9:45am approximately 1.5 h after feeding. Samples were pooled before transportation to the lab in thermo flasks, upon arrival flasks were transferred to an incubator at 39° C.

Anaerobic Van Soest buffer (2 L) was prepared the day before the experiment according to Goering and Van Soest (1970) (Tables 2.1 and 2.2).

The 36 extraction tubes containing dried down phospholipid residue (*Loliumperenne,* AberDart) were defrosted and then 5 mL strained rumen fluid was added (with continuous CO_2 gassing). Gassing with CO_2 was also used for the addition of anaerobic incubation buffer and enzyme stock solution. Due to the concentration levels of phospholipase being the prime factor, the amount of anaerobic buffer was modified in order to maintain the appropriate concentrations. The four treatments consisted of 75 μM, 100 μM, 125 μM and 150 μM concentrations, incubated up to 24 h in triplicate, with 0 h samples being used as controls (although these had phospholipase added but were frozen immediately). Tubes to contain 75 μM on enzyme were allocated 4.75 mL anaerobic buffer and 250 μL of 300 U/mL stock of A1; 100 μM tubes contained 4.67 mL buffer and 333 μL enzyme solution; 125 μM tubes with 4.58 mL buffer and 0.42 μL enzyme solution; 150 μM tube contained 4.5 mL buffer and 500 μL of enzyme solution.Tubes were vortexed gently to resuspend the phospholipids before incubation. After incubation at 39°C samples were destructively harvested by vortexing and a 2 mL subsample was taken at each time point and stored at -20°C for later RNA analysis. Samples for 0 h were subsampled straight away and then frozen at -20°C

3.2.9: Thin-Layer Gas Chromatography lipid fractionation

As described by Nichols in 1963, lipids were fractionated into four classes: the polar fraction (PF), monoacylglycerols and diacylglycerols (DAG), free fatty acids (FFA) and triacylglycerols (TAG), using organic solvent chloroform: methanol/isopropanol. The resulting lipids were then transformed into fatty acid methyl ester (FAME) for analysis using gas chromatography (GC) (Sukhija&Palmquist, 1988).

During analysis, two mobile phase tanks and a fume cupboard were used. To ensure full saturation inside the tanks, 50 mL of the mobile phase (2 mL acetic acid, 30 mL diethyl ester and 70 mL n-hexane) was added along with a saturation pad. The lid was then closed and the tank allowed to stand for 20 mins. The level of the mobile phase in the tanks was always below the PF on the TLC plates to ensure no contamination.

Polyester plates with silica gel PolyGram TLC prepared surfaces (36) were prepared by etching pencil lines to create two distinct sections. In chronological order, the PF was distributed evenly along the allocated section. Each application was allowed to dry before another layer was added, continuing until 1 mL of sample was loaded onto the plate. The plate was then allowed to dry in the fume cupboard and the procedure repeated for samples 2-6 (maximum 6 plates at a time). Pre-made standards for FFAs, DAGs and TAGs (20 mg/mL $CHCl_3$) were spotted into the left hand column etched onto the plates, which were then suspended in the tanks and lidded. The plates were left for approximately 1 h until the front of the mobile phase had travelled up, leaving a 1 inch gap at the top.

The plates were removed from the tanks and allowed to dry in the fume cupboard for 10 minutes, after which they were sprayed with 2,7-dichloroflourescin (100 mg in 100 mL isopropanol) and allowed to dry in the fume cupboard in the dark for 30 minutes. The plates were then examined under UV light and the front of the polar fraction was marked in pencil, along with that of the FFAs, DAGs and TAGs using the standards. Clean tubes (24) were assembled and labelled for each fraction, for each sample and the pre-marked lipid bands were scraped into the corresponding tube. Toluene (2 mL) containing internal standard (C23, 4 mg/mL) was added to each followed by 3 mL methanolic hydrochloric acid solution (5% HCl in methanol). Samples were flushed with nitrogen, and capped using PTFE-lined lids then vortexed gently to ensure thorough mixing. Samples were then placed in a water bath at 70°C for 2 h with intermittent hand mixing to ensure even distribution. Tubes were allowed to cool in the fume cupboard before 5 mL 6% potassium carbonate was slowly added (to avoid foaming) and then 2 mL toluene (no internal standard). Samples were vortexed vigorously and then centrifuged for 5 minutes at 1200 x g (Beckman J6-B, swing bucket rotor).

A new set of clean tubes was prepared as before containing 1 g anhydrous sodium sulphide, and for PF and DAG samples 1.5 g laboratory standard charcoal was also added (Sigma-Aldrich, UK). The top layer of toluene containing the lipids was then removed and placed into the new set of tubes using a Pasteur pipette. The tubes were then set to shake (KikaLaborTechnik KS501 digital) for 10 minutes to ensure thorough mixing and even distribution. Once again, the samples were centrifuged (1200 x g, 5 minutes) and the top layer of FFA and TAG samples were transferred into pre-labelled GC vials using a Pasteur pipette. For samples containing charcoal (PF and DAGs), the top layer was pipetted into labelled plasma tubes (with funnelled stems to collect the charcoal) which were centrifuged at 1000 x g for 3 minutes. The top, clear layer was then pipetted into pre-labelled GC vials. All vials were capped using a crimper and then stored at -20°C ready for GC analysis.

This process was repeated for the remainder of samples.

3.2.10: Total lipid methylation

This methylation technique was first described by Kramer and Zhou (2001) and was selected due to its suitability for transferring phospholipids and O-acyl lipids, such as free fatty acids, diacylglycerols and triacylglycerols.

The second set of samples was dried down under nitrogen using a dry block (DB-3D) at 50°C. In the fume cupboard, samples were re-suspended in 1 mL heptane and 4 mL 0.5 M sodium methoxide/methanol and hand mixed. Tubes were capped with PTFE-lined lids and placed in a water bath at 50°C for 15 minutes. They were allowed to cool in the fume cupboard with gentle hand mixing. Then, 4 mL acetyl chloride/methanol was added to each and the tubes were again placed into the water bath at 60°C for 1h.

The tubes were once again allowed to cool inside the fume cupboard and then 2 mL heptane and 2 mL dH_2O was added before being vigorously vortexed. The samples were next centrifuged at 2000 x g for 5 minutes, and then the top layer transferred into appropriately labelled GC vials using a Pasteur pipette. The vials were capped and crimped and then stored at - 20°C for GC analysis.

3.2.11: Statistical Analysis

Using Genstat (13^{th} Edition) an ANOVA was run to analyse the effects of A1 concentration upon fatty acid composition, Duncan's multiple range test was also included to provide significance levels for the differences between concentrations.

3.3: Results

3.3.1: Protein concentration

Original protein concentrations of Pl1 and Pl2ss following extraction and purification were too low for use in incubations with both the Microplate reader (Table 3.6) and Bradford assay (Table 3.7).

Table 3.6: Protein concentrations for Pl1 and Pl2ss as determined by the Microplate spectrophotometer, values shown are means of triplicate incubations (Biotek, UK).

Protein	Protein (mg/mL)	Protein (mg/mL)	Protein (mg/mL)	Average protein (mg/mL)
Pl1	0.463	0.459	0.450	0.4573
Pl2ss	0.559	0.563	0.557	0.5596

Table 3.7: Concentrations of Pl1 and Pl2ss using the Bradford Assay, showingthe mean of triplicate incubations and undiluted protein concentration

Protein	A_{595}	A_{595}	A_{595}	Average A_{595}	Undiluted protein concentration (mg/mL)
Pl1	0.232	0.230	0.227	0.229	0.4539
Pl2ss	0.278	0.281	0.279	0.279	0.5593

3.3.2: SDS PAGE of purified and concentrated samples

Coomassie stained 1DE gels revealed banding at the 75-85 kDa level for Pl1 and at the 60-70 kDa point for Pl2ss. This is in keeping with target protein's sizes (Privé, 2011). There was very little contamination, as demonstrated by the clear, singular bands in fig 3.1.

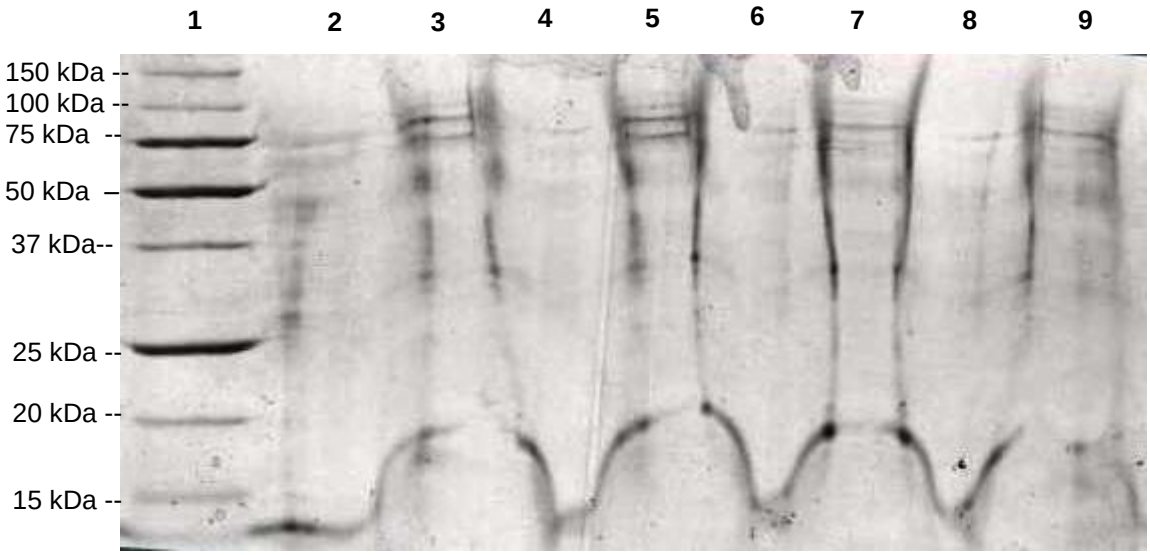


Fig 3.1: Analysis of Pl1 and Pl2ss expressed in E.coli Top10 cells following purification on a 12.5% Coomassie stained polyacrylamide gel. Lane 1 contains the 250 kDa Precision Plus Protein standard (Bio-Rad, UK); lanes 3 and 5 contain Pl1; lanes 7 and 9 contain Pl2ss.

After concentrating, protein content was significantly higher, however the volume obtained afterwards was less than 1/10th of the original volume, such that there was not enough

volume to test experimentally (Table 3.8).

Table 3.8: Concentrations of Pl1 and Pl2ss(in triplicate) and mean content after concentration procedure compared to previous content.

Protein	Conc. (mg/mL)	Conc. (mg/mL)	Conc. (mg/mL)	Avg. Conc. (mg/mL)	Avg. before (mg/mL)
Pl1	1.249	1.229	1.233	1.237	0.4556
Pl2ss	1.059	1.061	1.211	1.110	0.5595

3.3.4: Enzyme activity

Quantitative lipase activity is shown in Table 3.9, demonstrating low activity levels.

Table 3.9: Enzyme activity for Pl1 and Pl2ss determined using the average absorbancey at 410 nm.

Protein	A_{410}	A_{410}	A_{410}	Avg. A_{410}	Activity (µM PNP released/mL)
Pl1	0.146	0.142	0.139	0.142	5.950
Pl2ss	0.228	0.230	0.239	0.232	3.252

3.3.5: Changes to lipid profiles over incubation time

Fatty acids within total lipids

Addition of 75 and 100µM concentrations of phospholipase A1 did not generally result in substantial changes in any fatty acids analysed over time according to the Duncan's post-hoc test ($P<0.05$) (Table 3.10). Addition of 125 µM phospholipase A1 resulted in increases in all fatty acids analysed, except for BOCs, C18 and LCPUFA which did not show any significant changes ($P>0.05$) (Table 3.10). Addition of 150µM phospholipase A1 did, however, result in decreases ($P<0.05$) in all fatty acids analysed in comparison with 0h levels (Table 3.10). It should also be noted that for all fatty acid results significant variation is seen within 0h samples, probably as a consequence that phospholipases were added to these pre-freezing, thus these are not strictly 0h samples as 5-10 min lapsed before they were able to be frozen.

Fatty acids within the polar fraction

If phospholipase A1 conducted effective lipolysis of the phospholipids within the incubations, then a decrease in C16:0 should be apparent in the polar fraction, with an increase in the free fatty acid fraction subsequently (Table 3.11). Linoleic and Linolenic acid should also decrease within the polar fraction but increases may not be apparent in the free fatty acid fraction potentially due to biohydrogenation. C16:0 did not decrease following addition of 75, 100 and 125µM phospholipase A1 over time, nonetheless significant decreases ($P<0.05$) were seen upon addition of 150µM phospholipase A1 indicative that effective lipolysis had occurred upon addition of this concentration of the phospholipase (decrease by 6.47% over 24 h) (Table 3.11). Linoleic and linolenic acid concentrations did however decrease with the addition of all concentrations of phospholipase A1, indicative that the phospholipase may be more efficient at cleaving these fatty acids (decreases of 6.33% and 33.3% respectively) (Table 3.1).

Fatty acids within the free fatty acid fraction

In line with the C16:0 data in the polar fraction, increases in this lipid are seen within the free fatty acid fraction over time following 150µM addition of phospholipase A1 (increase of 5.74% over 24 h) (Table 3.12). It is also true that increases in C16:0 within this fraction over time were seen following additions of 100 and 125µM (4.85% and 8.53% respectively over 24 h) (Table 3.12). Levels of linolenic and linoleic acid increase with time following additions of 100, 125 and 150µM ($P<0.05$) (Table 3.12).At 100, 125 and 150µM there is an increase in levels of linoleic and linolenic acid: by 22.92%, 15.59% and 7.96% respectively, linolenic acid increased by 3.58% at 125µM and by 8.53% at 150µM. C18:1, *trans*-11 levels show a significant increase ($P>0.05$) at 75µM and 24 h (of 20%), and at 125 and 150µM at 4 h (of 4.45% and2% respectively) (table 3.12). There are also significant decreases ($P>0.05$) in 18:0 with both time and concentration (125µM 18:0 content decreases by 8.53% and from 75 to 150µM, 24 h content is reduced by 3.46%)(Table 3.12).

Fatty acids in the diacylglycerol fraction

C16:0 and C18:0 percentages decline significantly ($P>0.05$), reducing by 14.29% and 3.72% respectively, with concentration of A1 phospholipase added as they move into the FFA fraction (Table 3.13). C18:1, *trans*-11 content decreases over concentration, as more is moving into the FFA fraction (Table 3.13). The same can be said for linoleic and linolenic acid, which decrease over concentration. Variation in the 0h samples can be attributed to the

fact that phospholipase A1 was added before freezing, which can take up to 10 mins allowing processes to continue (Table 3.13).

Fatty acids in the triacylglycerol fraction

Similar to the diacylglyerol fraction, C16 and C18 content generally decreases, along with declining linoleic and linolenic acid levels (Table 3.14). Conversely, there is an increase in C18:1, *trans*-11 content, values reaching 20% at 125 μM at 24 h, corresponding to values found in the free fatty acid fraction (Table 3.14).

Table 3.10: Fatty acids with in total lipids (as percentage of total fatty acids) in the presence of different concentrations of A1 phospholipase (75µM, 100µM, 125µM and 150 µM,) incubated *in vitro* up to 24 h**.**

Conc (µM)	Time (h)	C12	C14	BOCs	C16	C18	C18:1, *trans*-10	C18:1, *trans*-11	C18:1, *cis*-11	18:2, *cis*-9, *cis*-12	18:3 *n*-3	18:2, *cis*-9,*trans*-11	Sum C18 *cis*	Sum C18 *trans*	LCPUFA
75	0	0.28^{a}	0.75^{a}	1.09^{a}	10.97^{b}	5.22^{a}	0.04^{a}	0.47^{a}	0.02^{a}	7.33^{a}	30.57^{b}	0.06^{a}	7.70^{a}	1.66^{a}	1.39^{a}
	4	0.34^{a}	0.90^{a}	1.18^{a}	13.17^{b}	6.60^{a}	0.05^{a}	0.56^{a}	0.24^{a}	8.80^{a}	36.70^{b}	0.07^{a}	9.24^{ab}	1.99^{a}	1.67^{a}
	24	0.31^{a}	0.84^{a}	1.18^{a}	12.32^{b}	5.86^{a}	0.05^{a}	0.52^{a}	0.23^{a}	8.24^{a}	34.34^{b}	0.07^{a}	8.64^{ab}	1.864^{a}	1.56^{a}
	SED	0.05	0.13	0.18	1.93	0.95	0.01	0.08	0.01	1.29	5.39	0.01	1.36	0.29	0.24
	P	<.001	<.001	<.001	0.016	0.255	<.001	<.001	<.001	0.432	0.013	<.001	0.090	<.001	<.001
100	0	0.32^{a}	0.85^{a}	1.12^{a}	12.39^{b}	6.06^{a}	0.05^{a}	0.53^{a}	0.23^{a}	8.29^{a}	34.55^{b}	0.07^{a}	8.70^{ab}	1.88^{a}	1.57^{a}
	4	0.28^{a}	0.74^{a}	1.08^{a}	10.87^{c}	5.17^{a}	0.04^{a}	0.46^{a}	0.20^{a}	7.27^{a}	30.31^{b}	0.06^{a}	7.63^{a}	1.65^{a}	1.38^{a}
	24	0.36^{a}	0.95^{a}	1.37^{a}	13.95^{b}	6.64^{a}	0.05^{a}	0.59^{a}	0.26^{a}	9.33^{a}	38.89^{b}	0.08^{a}	9.79^{ab}	2.11^{a}	1.77^{a}
	SED	0.055	0.146	0.216	2.047	1.099	0.008	0.091	0.040	1.427	5.950	0.012	1.498	0.3231	0.271
	P	<.001	<.001	<.001	0.0014	0.307	<.001	<.001	<.001	0.311	0.015	<.001	0.098	<.001	<.001
125	0	0.26^{a}	0.69^{a}	1.02^{a}	10.10^{b}	5.81^{a}	0.04^{a}	0.43^{a}	0.19^{a}	6.75^{a}	28.15^{b}	0.06^{a}	7.09^{a}	1.53^{a}	1.58^{a}
	4	0.35^{b}	0.95^{b}	1.25^{a}	13.89^{c}	6.84^{a}	0.05^{b}	0.59^{b}	0.26^{b}	9.29^{b}	38.73^{c}	0.08^{b}	9.75^{b}	2.10^{b}	1.43^{a}
	24	0.34^{b}	0.90^{b}	1.19^{a}	13.14^{c}	6.25^{a}	0.05^{b}	0.56^{b}	0.25^{b}	8.78^{b}	36.63^{c}	0.07^{b}	9.22^{b}	1.99^{b}	1.60^{a}
	SED	0.032	0.085	0.193	1.253	1.117	0.005	0.053	0.023	0.838	3.492	0.007	0.879	0.190	0.195
	P	<.001	<.001	<.001	<.001	0.415	<.001	<.001	<.001	0.021	<.001	<.001	0.005	<.001	<.001
150	0	0.33^{b}	0.88^{b}	1.29^{b}	12.95^{c}	6.16^{b}	0.05^{b}	0.55^{b}	0.24^{b}	8.65^{b}	36.08^{c}	0.07^{b}	9.08^{b}	1.96^{b}	1.64^{b}
	4	0.30^{a}	0.78^{a}	1.09^{a}	11.40^{b}	5.42^{a}	0.04^{a}	0.48^{a}	0.21^{a}	7.62^{a}	31.78^{b}	0.06^{a}	8.00^{a}	1.73^{a}	1.45^{a}
	24	0.28^{a}	0.75^{a}	1.02^{a}	10.97^{b}	5.22^{a}	0.04^{a}	0.47^{a}	0.20^{a}	7.34^{a}	30.59^{b}	0.06^{a}	7.70^{a}	1.66^{a}	1.39^{a}
	SED	0.013	0.035	0.0773	0.513	0.244	0.002	0.022	0.010	0.343	1.430	0.003	0.36	0.078	0.065
	P	<.001	<.001	<.001	<.001	0.008	<.001	<.001	<.001	0.006	<.001	<.001	<.001	<.001	<.001

A two-way ANOVA was conducted upon raw data to assess the effect of different concentrations on the fraction's fatty acids (Appendix CD). SED: Standard Error of Differences of means, P: significance of effect of A1 concentration and incubation time (P=<0.05). Values within the same row were allocated different superscripts where significantly different.

Table 3.11: Fatty acids with in the polar fraction (as percentage of total fatty acids) in the presence of different concentrations of A1 phospholipase (75µM, 100µM, 125µM and 150 µM,) incubated *in vitro* up to 24 h**.**

Conc (µM)	Time (h)	C12	C14	BOCs	C16	C18	C18:1, *trans*-10	C18:1, *trans*-11	C18:1, *cis*-11	18:2, *cis*-9, *cis*-12	18:3 *n*-3	18:2, *cis*-9,*trans*-11	Sum C18 *cis*	Sum C18 *trans*	LCPUFA
75	**0**	0.17^{a}	0.85^{a}	1.65^{a}	17.46^{b}	1.41^{a}	0.00^{a}	0.28^{a}	0.32^{a}	12.43^{b}	58.24^{a}	0.35^{a}	1.58^{a}	2.00^{a}	59.08^{d}
	4	0.29^{a}	1.36^{ab}	2.91^{b}	20.40^{c}	2.25^{a}	0.00^{a}	0.76^{ab}	0.47^{b}	12.63^{b}	49.02^{b}	0.48^{a}	3.71^{b}	1.77^{a}	50.04^{c}
	24	0.73^{b}	2.83^{c}	4.54^{c}	22.47^{d}	4.15^{b}	0.04^{a}	3.38^{bc}	0.70^{c}	10.12^{a}	33.17^{c}	0.79^{b}	1.25^{a}	2.33^{a}	46.77^{b}
	SED	0.141	0.444	0.687	0.889	0.804	0.049	1.586	0.086	1.150	1.607	0.138	1.196	1.764	3.88
	P	0.002	0.004	0.006	<.001	0.006	<.001	0.017	<.001	0.029	0.0469	<.001	<.001	0.003	<.001
100	**0**	0.20^{a}	1.20^{a}	2.47^{a}	18.84^{b}	1.69^{a}	0.00^{a}	0.44^{a}	0.38^{a}	12.91^{b}	58.21^{d}	0.00	2.05^{c}	1.82^{a}	59.49^{c}
	4	0.31^{a}	1.52^{a}	3.28^{a}	21.86^{b}	2.77^{a}	0.04^{a}	0.94^{a}	0.52^{b}	13.34^{b}	50.15^{c}	0.00	2.25^{a}	1.68^{a}	52.10^{c}
	24	1.08^{b}	4.17^{b}	7.31^{b}	26.04^{c}	7.59^{b}	0.26^{a}	5.45^{b}	1.01^{c}	7.20^{a}	33.10^{b}	0.00	3.23^{a}	2.70^{a}	32.96^{b}
	SED	0.096	0.399	0.806	1.779	1.66	0.155	1.830	0.0795	0.830	4.590	N/A	0.2193	1.252	4.830
	P	<.001	<.001	<.001	<.001	0.010	<.001	0.013	<.001	<.001	<.001	N/A	<.001	<.001	<.001
125	**0**	0.21^{a}	1.10^{a}	0.63	18.95^{b}	1.72^{a}	0.00	0.46^{a}	0.40^{a}	12.99^{c}	58.38^{c}	0.00	1.38^{d}	4.64^{a}	3.16^{b}
	4	0.24^{a}	1.06^{a}	0.86	20.30^{c}	2.83^{a}	0.00	0.49^{a}	0.40^{a}	12.51^{d}	52.54^{c}	0.00	2.28^{c}	13.11^{a}	1.21^{a}
	24	1.15^{c}	4.36^{c}	0.84	27.00^{d}	4.76^{b}	0.00	2.73^{b}	0.96^{b}	9.85^{b}	43.65^{b}	0.00	0.98^{a}	7.10^{a}	1.20^{a}
	SED	0.022	0.143	0.090	0.543	0.107	N/A	0.092	0.024	0.248	1.227	N/A	0.066	0.149	0.907
	P	<.001	<.001	<.001	<.001	<.001	N/A	<.001	<.001	<.001	<.001	N/A	0.025	<.001	<.001
150	**0**	0.12^{a}	0.85^{a}	0.67^{a}	12.34^{b}	1.00^{a}	0.00	0.20^{a}	0.30^{a}	8.64^{b}	41.19^{a}	0.10^{a}	1.42^{a}	4.03^{a}	0.88^{a}
	4	0.27^{a}	0.21^{a}	0.82^{a}	11.25^{b}	1.08^{a}	0.00	0.30^{a}	0.56^{a}	7.31^{b}	29.31^{b}	0.11^{a}	1.36^{a}	4.73^{a}	0.97^{a}
	24	1.14^{b}	0.69^{b}	1.74^{b}	5.87^{a}	1.06^{a}	0.00	0.56^{b}	2.53^{b}	2.31^{a}	7.89^{c}	0.30^{b}	0.91^{a}	2.09^{a}	2.18^{b}
	SED	0.082	0.117	0.1937	0.601	0.052	N/A	0.075	0.507	0.071	1.010	0.064	0.152	0.685	0.304
	P	<.001	<.001	<.001	<.001	<.001	N/A	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001

A two-way ANOVA was conducted upon raw data to assess the effect of different concentrations on the fraction's fatty acids (Appendix CD). SED: Standard Error of Differences of means, P: significance of effect of A1 concentration and incubation time (P=<0.05). Values within the same row were allocated different superscripts where significantly different. Where values are not allocated superscripts, Duncan's multiple range tests could not be carried out.

Table 3.12: Fatty acids with in the free fatty acid fraction (as percentage of total fatty acids) in the presence of different concentrations of A1 phospholipase (75µM, 100µM, 125µM and 150 µM,) incubated *in vitro* up to 24 h**.**

Conc (µM)	Time (h)	C12	C14	BOCs	C16	C18	C18:1, *trans*-10	C18:1, *trans*-11	C18:1, *cis*-11	18:2, *cis*-9, *cis*-12	18:3 *n*-3	18:2, *cis*-9,*trans*-11	Sum C18 *cis*	Sum C18 *trans*	LCPUFA
75	**0**	0.78^{c}	1.17^{b}	0.86^{a}	14.23^{b}	23.82^{c}	0.10^{a}	2.01^{a}	0.18^{a}	5.32^{a}	20.54^{c}	0.00^{a}	5.40^{b}	3.31^{a}	3.37^{a}
	4	0.36^{b}	0.72^{a}	0.82^{a}	15.14^{b}	16.42^{bc}	0.30^{a}	11.91^{ab}	0.25^{a}	3.15^{a}	9.44^{b}	1.09^{b}	3.32^{ab}	14.70^{ab}	2.02^{a}
	24	0.22^{a}	0.59^{a}	0.91^{a}	15.38^{b}	10.05^{ab}	0.34^{a}	22.15^{b}	0.28^{a}	0.49^{a}	1.13^{a}	0.20^{a}	0.64^{a}	24.23^{b}	4.46^{a}
	SED	0.055	0.104	0.197	1.761	4.414	0.141	7.46	0.086	0.572	4.560	0.345	1.583	7.840	2.133
	P	<.001	<.001	<.001	0.003	0.011	<.001	0.045	<.001	<.001	0.006	<.001	0.002	0.050	0.008
100	**0**	0.67^{c}	0.90^{c}	0.73^{a}	11.74^{b}	17.01^{c}	0.09^{a}	1.66^{a}	0.17^{a}	1.63^{b}	20.96^{d}	0.00^{a}	0.80^{a}	4.01^{a}	21.61^{c}
	4	0.44^{b}	0.79^{b}	0.83^{a}	15.26^{c}	17.10^{c}	0.21^{a}	5.19^{b}	0.25^{b}	5.08^{b}	14.00^{c}	1.54^{b}	1.94^{b}	8.71^{b}	14.51^{b}
	24	0.24^{a}	0.63^{a}	1.09^{b}	16.59^{c}	11.12^{b}	0.43^{b}	0.41^{a}	0.31^{c}	24.55^{a}	24.54^{c}	0.10^{a}	2.88^{c}	28.07^{d}	14.00^{b}
	SED	0.027	0.064	0.087	0.815	1.036	0.074	1.452	0.031	0.536	1.353	0.156	0.245	1.451	1.865
	P	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
125	**0**	0.33^{b}	0.48^{a}	0.48^{a}	6.75^{b}	13.13^{c}	0.06^{a}	1.03^{a}	0.08^{a}	1.03^{c}	12.61^{bc}	0.00^{a}	5.49^{c}	1.66^{a}	51.89^{d}
	4	0.32^{b}	0.72^{b}	0.72^{b}	12.80^{c}	8.47^{b}	0.18^{b}	5.48^{b}	0.19^{b}	5.48^{b}	14.98^{c}	1.27^{c}	3.03^{b}	7.81^{b}	14.59^{a}
	24	0.14^{a}	0.48^{a}	0.48^{a}	15.28^{d}	7.84^{b}	0.22^{b}	0.39^{a}	0.23^{c}	15.89^{a}	15.80^{c}	0.13^{b}	0.73^{a}	17.43^{c}	28.10^{c}
	SED	0.016	0.032	0.032	0.692	0.567	0.045	2.38	0.018	0.372	1.654	0.049	0.424	2.555	2. 882
	P	<.001	<.001	<.001	<.001	<.001	<.001	0.002	<.001	<.001	<.001	<.001	<.001	0.002	<.001
150	**0**	0.33^{c}	0.49^{a}	0.35^{a}	6.20^{b}	7.67^{ab}	0.05^{a}	0.83^{a}	0.09^{a}	2.87^{c}	10.75^{c}	0.00^{a}	0.97^{a}	4.01^{a}	23.59^{c}
	4	0.21^{b}	0.43^{a}	0.36^{a}	8.73^{c}	7.02^{b}	0.08^{a}	2.34^{b}	0.12^{a}	3.93^{b}	6.85^{b}	0.16^{b}	3.42^{b}	8.71^{b}	16.11^{b}
	24	0.15^{a}	0.45^{a}	0.81^{b}	11.94^{d}	6.59^{ab}	0.19^{b}	0.37^{a}	0.16^{b}	10.83^{a}	10.53^{d}	0.91^{c}	6.96^{c}	28.07^{d}	4.13^{a}
	SED	0.028	0.039	0.066	0.644	1.041	0.024	0.436	0.019	0.194	0.995	0.040	0.477	1.451	1.954
	P	<.001	<.001	<.001	<.001	0.122	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001

A two-way ANOVA was conducted upon raw data to assess the effect of different concentrations on the fraction's fatty acids (Appendix CD). SED: Standard Error of Differences of means, P: significance of effect of A1 concentration and incubation time (P=<0.05). Values within the same row were allocated different superscripts where significantly different. Where values are not allocated superscripts, Duncan's multiple range tests could not be carried out.

Table 3.13: Fatty acids with in the diacylglycerol fraction (as percentage of total fatty acids) in the presence of different concentrations of A1 phospholipase (75µM, 100µM, 125µM and 150 µM,) incubated *in vitro* up to 24 h**.**

Conc (µM)	Time (h)	C12	C14	BOCs	C16	C18	C18:1, *trans*-10	C18:1, *trans*-11	C18:1, *cis*-11	18:2, *cis*-9, *cis*-12	18:3 *n*-3	18:2, *cis*-9,*trans*-11	Sum C18 *cis*	Sum C18 *trans*	LCPUFA
	0	0.77^{b}	1.98^{c}	0.42^{a}	17.08^{b}	8.12^{b}	0.00^{a}	0.08^{a}	0.42^{b}	12.96^{c}	46.55^{c}	0.27^{a}	0.16^{a}	3.05^{a}	72.71^{bc}
75	**4**	0.93^{b}	1.38^{b}	0.41^{a}	16.77^{b}	4.81^{a}	0.11^{a}	4.24^{a}	0.28^{a}	10.95^{c}	26.39^{b}	0.99^{a}	0.12^{a}	4.17^{b}	76.88^{c}
	24	0.34^{a}	0.65^{a}	0.23^{a}	4.70^{a}	3.31^{a}	0.15^{a}	5.79^{a}	0.22^{a}	2.89^{a}	3.34^{a}	1.33^{a}	0.86^{b}	8.49^{c}	66.74^{b}
	SED	0.180	0.119	0.185	0.737	1.312	0.138	3.880	0.080	1.244	7.55	0.792	0.123	0.520	3.750
	P	0.013	<.001	<.001	<.001	0.011	<.001	0.231	<.001	<.001	0.001	<.001	<.001	<.001	<.001
	0	0.31^{a}	0.72^{a}	0.42^{a}	16.96^{c}	2.63^{a}	0.00^{a}	0.07^{a}	0.18^{ab}	5.76^{c}	14.54^{d}	0.74^{a}	0.12^{a}	2.24^{a}	80.52^{bc}
100	**4**	0.30^{a}	0.69^{a}	0.41^{a}	16.21^{b}	2.89^{a}	0.00^{a}	0.20^{a}	0.12^{a}	4.78^{b}	12.28^{c}	0.11^{a}	0.16^{a}	4.04^{b}	78.20^{c}
	24	0.29^{a}	0.57^{a}	0.23^{a}	4.39^{a}	2.55^{a}	0.21^{b}	6.09^{b}	0.27^{b}	1.88^{a}	2.22^{a}	0.04^{a}	0.86^{b}	5.34^{c}	68.74^{b}
	SED	0.054	0.093	0.185	0.376	0.293	0.048	0.141	0.056	0.373	0.579	0.594	0.123	0.520	3.752
	P	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
	0	0.36^{b}	0.30^{a}	0.08^{a}	5.34^{b}	1.85^{c}	0.00^{a}	0.08^{a}	0.08^{a}	4.29^{b}	9.83^{b}	0.00^{a}	1.68^{a}	0.80^{a}	77.91^{b}
125	**4**	0.19^{a}	0.40^{a}	0.39^{b}	4.41^{b}	1.53^{b}	0.00^{a}	0.15^{a}	0.08^{a}	3.02^{b}	9.03^{b}	0.00^{a}	3.09^{b}	0.88^{a}	78.30^{b}
	24	0.14^{a}	0.62^{b}	0.39^{b}	2.78^{a}	1.04^{a}	0.08^{b}	2.99^{b}	0.11^{a}	1.55^{a}	1.78^{a}	0.25^{b}	4.35^{b}	4.10^{b}	72.52^{b}
	SED	0.052	0.062	0.084	0.682	0.089	0.008	0.729	0.054	0.793	0.959	0.056	0.770	0.680	4.100
	P	<.001	<.001	<.001	0.016	<.001	<.001	<.001	<.001	0.001	<.001	<.001	<.001	<.001	<.001
	0	0.19^{a}	0.42^{a}	0.12^{a}	4.63^{b}	1.27^{a}	0.00^{a}	0.05^{a}	0.08^{a}	4.13^{b}	9.24^{c}	0.00^{a}	81.76^{b}	0.26^{a}	0.59^{a}
150	**4**	0.19^{a}	0.30^{a}	0.00^{a}	2.48^{a}	1.09^{a}	0.00^{a}	0.08^{a}	0.04^{a}	1.84^{a}	4.78^{b}	0.03^{a}	86.32^{bc}	0.14^{a}	0.58^{a}
	24	0.16^{a}	0.34^{a}	0.16^{a}	2.04^{a}	0.98^{a}	0.02^{a}	1.50^{b}	0.06^{a}	1.12^{a}	1.63^{a}	0.10^{a}	89.09^{c}	2.21^{b}	0.48^{a}
	SED	0.042	0.118	0.110	0.546	0.570	0.021	0.490	0.048	0.603	0.781	0.117	3.329	0.661	0.348
	P	<.001	<.001	<.001	0.002	0.002	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001

A two-way ANOVA was conducted upon raw data to assess the effect of different concentrations on the fraction's fatty acids (Appendix CD). SED: Standard Error of Differences of means, P: significance of effect of A1 concentration and incubation time (P=<0.05). Values within the same row were allocated different superscripts where significantly different. Where values are not allocated superscripts, Duncan's multiple range tests could not be carried out.

Table 3.14: Fatty acids with in the triacylglycerol fraction (as percentage of total fatty acids) in the presence of different concentrations of A1 phospholipase (75µM, 100µM, 125µM and 150 µM,) incubated *in vitro* up to 24 h**.**

Conc (µM)	Time (h)	C12	C14	BOCs	C16	C18	C18:1, *trans*-10	C18:1, *trans*-11	C18:1, *cis*-11	18:2, *cis*-9, *cis*-12	18:3 *n*-3	18:2, *cis*-9,*trans*-11	Sum C18 *cis*	Sum C18 *trans*	LCPUFA
	0	0.79^{b}	1.41^{b}	0.87^{b}	17.50^{b}	4.93^{a}	0.00^{a}	0.08^{a}	0.29^{a}	13.27^{c}	1.20^{a}	1.46^{a}	0.29^{a}	9.19^{a}	9.03^{b}
75	**4**	0.98^{b}	2.10^{c}	0.80^{b}	17.75^{b}	8.61^{b}	0.12^{a}	4.50^{a}	0.45^{b}	13.71^{c}	2.33^{b}	2.80^{b}	0.42^{a}	13.28^{a}	31.19^{a}
	24	0.35^{a}	0.67^{a}	0.37^{a}	4.85^{a}	3.42^{a}	0.15^{a}	5.98^{a}	0.23^{a}	2.99^{a}	0.97^{a}	1.22^{a}	0.18^{a}	9.86^{a}	65.58^{c}
	SED	0.194	0.136	0.215	0.782	1.41	0.145	4.090	0.081	1.249	0.331	0.373	0.213	10.69	7.870
	P	0.014	<.001	<.001	<.001	0.010	<.001	0.248	<.001	<.001	<.001	<.001	<.001	0.939	0.001
	0	0.79^{b}	1.41^{b}	0.87^{b}	17.50^{b}	4.93^{a}	0.00^{a}	0.08^{a}	0.29^{a}	14.96^{c}	1.20^{a}	1.20^{a}	1.46^{a}	0.29^{a}	9.19^{a}
100	**4**	0.99^{b}	2.10^{c}	0.80^{b}	17.75^{b}	8.61^{b}	0.12^{a}	4.50^{a}	0.45^{b}	13.21^{c}	2.33^{b}	2.33^{b}	2.80^{b}	0.42^{a}	13.28^{a}
	24	0.35^{a}	0.67^{a}	0.37^{a}	4.85^{a}	3.42^{a}	0.15^{a}	5.98^{a}	0.23^{a}	5.95^{a}	0.97^{a}	0.97^{a}	1.22^{a}	0.18^{a}	9.86^{a}
	SED	0.194	0.136	0.215	0.782	1.410	0.145	4.090	0.081	1.249	0.331	0.331	0.373	0.213	10.690
	P	0.014	<.001	<.001	<.001	0.010	<.001	0.248	<.001	<.001	<.001	<.001	<.001	<.001	0.939
	0	0.72^{b}	1.53^{b}	0.00^{a}	22.27^{c}	5.25^{b}	0.00^{a}	0.38^{a}	0.42^{b}	15.20^{c}	14.66^{b}	1.68^{b}	0.00	0.15^{a}	3.67^{a}
125	**4**	0.38^{a}	0.66^{a}	0.00^{a}	21.67^{c}	1.63^{a}	0.00^{a}	0.16^{a}	0.08^{a}	14.56^{c}	9.58^{a}	0.53^{a}	0.00	0.12^{a}	2.68^{a}
	24	1.32^{d}	2.84^{c}	0.17^{a}	20.00^{b}	13.42^{c}	0.54^{b}	20.67^{b}	0.80^{c}	10.95^{b}	12.65^{b}	4.77^{c}	0.00	0.18^{a}	8.96^{a}
	SED	0.104	0.232	0.125	1.023	1.139	0.006	2.732	0.098	1.113	1.114	0.470	N/A	0.120	5.530
	P	<0.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	N/A	<.001	0.252
	0	0.90^{a}	0.00^{a}	0.18^{a}	2.36^{a}	0.53^{b}	0.07^{a}	0.07a	0.22^{a}	16.00^{a}	14.56^{a}	1.47^{a}	1.40^{a}	0.12^{a}	4.23^{a}
150	**4**	0.06^{ab}	0.00^{a}	0.11^{a}	0.71^{a}	0.28^{b}	0.07^{a}	0.07a	0.01^{a}	14.69^{a}	5.12^{a}	1.30^{a}	1.58^{a}	0.09^{a}	6.08^{ab}
	24	1.09^{b}	0.14^{a}	0.22^{a}	4.37^{a}	2.13^{b}	0.07^{a}	6.33c	3.67^{b}	10.56^{a}	6.53^{a}	2.87^{a}	0.98^{a}	1.23^{a}	8.84^{bc}
	SED	0.433	0.162	0.171	0.947	2.689	0.009	1.864	0.355	1.092	2.875	0.659	0.125	0.460	2.515
	P	0.056	<.001	0.001	0.024	0.003	0.042	<.001	<.001	<.001	<.001	<.001	<.001	0.292	0.032

A two-way ANOVA was conducted upon raw data to assess the effect of different concentrations on the fraction's fatty acids (Appendix CD). SED: Standard Error of Differences of means, P: significance of effect of A1 concentration and incubation time (P=<0.05). Values within the same row were allocated different superscripts where significantly different. Where values are not allocated superscripts, Duncan's multiple range tests could not be carried out.

3.4: Discussion

This study investigated the potential of inhibiting biohydrogenation by increasing lipolysis through the release of potentially inhibitory concentrations of fatty acids from fresh forage phospholipids to the free fatty acid fraction. If levels of PUFA released from the polar fraction containing plant phospholipids can be enhanced to levels which inhibit biohydrogenation as analysed in chapter 2, then addition of phospholipases to the diet of forage fed cows may be an option for reducing saturated fatty acid levels in meat and milk.

Lipolysis and biohydrogenation

Forage phospholipids are typically rich in C16:0, linolenic and linoleic acid (Huws *et al.*, 2009). If phospholipase A1 were to enhance lipolysis then levels of C16:0, which is not transformed further, should decrease within the polar lipid fraction and increase within the free fatty acid fraction. Notable decreases in C16:0 were achieved using 150 μM phospholipase A1, suggesting that this higher concentration was more effective in enhancing lipolysis. C16:0 content in the free fatty acid fraction increased at 100, 125 and 150μM suggesting that lipolysis was successfully enhanced.

Linoleic and Linoleic acid in the polar fraction also decrease as they undergo lipolysis and become apparent in the free fatty acid fraction, however LA and LNA are not as reliable an indicator of lipolysis as C16 as they biohydrogenated.

In the free fatty acid fraction C16:0 content continues to increase suggesting efficient lipolysis, whilst C18:0 content decreases significantly ($P>0.05$). Linoleic and Linolenic acid values are also decreasing, which suggests biohydrogenationbut not fully to C18:0. C18:1, *trans*-11 which has high values particularly at 24 h, which mirrors the changes in LA and LNA. This trend became apparent from 100 μM concentrations of phospholipase, but 75μM had little effect. This accumulation of C18:1, *trans*-11 brings into question its potential toxicity to rumen microbes and its possible ability in suppressing biohydrogenation to C18:0. As this effect seems clearer with higher concentrations, it is feasible to suggest that the higher the concentrations of phospholipase A1, the more C18:1, *trans*-11 may accumulate and higher levels of inhibition of C18:0 conversion could occur.

The data for triacylglycerol and diacylglycerol fractions demonstrate a decrease in linoleic and linolenic acid as well as C16 and C18, thus these fractions would also contribute to the levels seen in the free fatty acid fraction. These fractions however, do not hold any

phospholipids, and are therefore more indicative of processes active within the rumen fluid and caused by the rumen microbiome.

Branched and odd chain fatty acids can be used as an indicator of the rumen microbial diversity (Vlaeminck*et al.*, 2006), which showed little changes in BOCs over time and concentration suggesting that there was no change in the bacterial diversity. Nonetheless due to time constraints TRFLP bacterial analysis was not undertaken.

Overall, lipolysis was enhanced to an extent, and in turn, biohydrogenation was partially inhibited. This was best achieved using higher (150 μM) concentrations of the phospholipase A1. There was a linear relationship between the perceived amount of lipolysis and concentration of A1. As previously mentioned, this is most probably due to the mixed population introduced in 50% fresh rumen fluid, including any lipid carry over, for future experiments, 10% rumen fluid may help results become clearer.The accumulation of C18:1, *trans*-11 is an interesting occurrence, as this transient PUFA is used by the Δ9-desaturase enzyme found in the ruminant mammary gland to endogenously produce CLA – a highly sought after health beneficial fatty acid (Griinari, 2000).Whilst these results are somewhat encouraging, this remains a novel method of inhibiting biohydrogenation, and in order to confirm results the experiment would need to be repeated, accompanied by further research into lipolysis and bacterial lipases.

So, whilst enhancement is a potential avenue for inhibition of biohydrogenation the process would also be extremely expensive and in order to achieve further inhibition would require even higher concentrations of phospholipase. With this in mind, returning to data generated by Huxley (2012), it is apparent that Pl2ss ranked the highest of the three selected enzymesin terms of activity and U/mg. The phospholipase used here also showed high activity, falling in the middle of the three enzymes with Pl1 showing the lowest values. Due to unsuccessful culturing of hosts and time constraints, Pl1 and Pl2ss were not used here, however a repeat study using the two phospholipases characterised from the rumen would be interesting – particularly to assess the efficacy of Pl2ss in enhancing lipolysis, compared to A1 (Privé, 2010).

It is also possible that inhibition of lipolysis may be more appropriate to cease provision of precursors for biohydrogenation altogether. Though this theory relies upon further, more in depth investigation of lipolysis and lipases of the rumen, which would be fundamental in the search for a chemical inhibitor.

Chapter 4: General discussion

There is a clear need to reduce the saturated fatty acid content of meat and milk. Indeed over 90% of ingested PUFA within the rumen are biohydrogenated to saturated fatty acids, resulting in ruminant products which are deemed unhealthy if not consumed in moderation. The majority of research directed into ruminal lipid metabolism focusses on the process of biohydrogenation, due to the fact that its intermediates (such as conjugated linoleic acids) and end products (SFAs) have the potential to affect human health. Nonetheless, attempts to beneficially manipulate biohydrogenation have to date been largely unsuccessful due to the complexity of the metabolic processes and the microbiota involved. Effective lipolytic release of fatty acids, coupled with their subsequent esterification, is required before biohydrogenation can occur. There is comparatively little research published concerning the process of lipolysis and only a few lipolytic organisms and their enzymes have been characterised. This leaves open a new, novel approach to potentially control biohydrogenation, through manipulating lipolysis. There is also the potential for bacterial lipases to be applied in industry, for example in biofuels. The end goals of this project were to ascertain the level of PUFA required to inhibit biohydrogenation and whether these levels can be achieved through addition of lipases.

This hypothesis was explored first through the use of a batch culture experiment, using different concentrations of linoleic and linolenic acid to assess levels required to inhibit biohydrogenation. Using FAME analysis, total lipids were quantified and variation between group means analysed using an ANOVA and Duncan's multiple range test. As previously indicated, this experiment was based upon on work published by Maia *et al.*(2007), where concentrations up to 50 µM PUFA were used.

The experiment showed that biohydrogenation was inhibited to an extent; however recovery occurred within 24 h. Over time, the reduction was limited to between 4 and 8 h, this is probably due to the fact that the PUFA was added and some was biohydrogenated over time, thus reducing its levels and allowing all bacteria that weren't initially affected to further biohydrogenated.

Contrary to the expected, there was not a linear relationship between LNA/LA concentration and PUFA/SFA content;instead the trend appeared more quadratic. Inhibition appeared to peak at 250 and 500 µM, contrary to the expected increase in inhibition with concentration. This warrants further investigation, perhaps using 10% rumen fluid which could help highlight differences in the fatty acid profiles and provide more data for metabolism in a mixed culture.

It also appeared that LA was more effective in inhibition than LNA; which is contradictory to previously published data which suggest LNA is more toxic to bacteria than LA (Maia *et al.*, 2007; Jenkins *et al.*, 2008). The mechanism of toxicity of PUFA to bacteria is as yet unconfirmed, although there are a few hypotheses. Following the theory that phospholipids are incorporated into the bacterial cell membrane, the larger the disruption of the membrane, the worse the damage would be (Jenkins, 1993; Khulusi*et al.*, 1995). So perhaps the position of the double bonds in LA causes larger disruption due to the angle of the kink created, where the double bonds in the structure of LNA cause the chain to fold in slightly closer.

HaeII-based RNA TRFLP suggested that the bacterial community of the rumen were unaffected during the observed changes; suggesting minimal toxicity of LA and LNA to the ruminal bacterial community. Nonetheless the use of next generation sequencing may show changes in the microbiome as the depth of information obtained by T-RFLP is limited. Experiments trialling the inclusion of fish oils and red clover showed substantial changes in the bacterial population, as fish oils tend to contain high levels of EPA, DHA and long chain fatty acids which are known for their toxicity to rumen bacteria (Kim *et al.*, 2008; Huws *et al.*, 2010). Probably due to their prevalence in forage and the ruminant diet LA and LNA are not as toxic to the bacterial population as EPA and DHA; as such, a similar experiment using EPA and DHA could provide interesting results.

The second part of the project addressed the hypothesis of whether phospholipases could enhance lipolysis to the point that PUFA build up would inhibit biohydrogenation. This was investigated using another batch culture with concentrations of A1 phospholipase (from *Thermomyceslaguninosus*) added in rumen fluid with their effects observed over time. Initially, rumen phospholipases Pl1 and Pl2ss characterised by Privé (2011), were also selected but due to time constraints and very low enzymatic activity associated with the initial culture, this was not possible. Instead the A1 phospholipase was used alone. Following harvesting over time fatty acids were then extracted and both TLC and total lipid analysis were carried out.

The resulting data suggested that lipolysis had been enhanced, and biohydrogenation had been partly inhibited following the addition of 150µM phospholipase. There were decreases in C16, linoleic and linolenic acid in the polar fraction, then increases in the free fatty acid fraction confirming efficient lipolysis. As linoleic and linolenic acid began to decrease in the FFA fraction, the expected corresponding increase in C18:0 did not occur, instead an increase in the biohydrogenation intermediate C18:1, *trans*-11 was apparent.This indicated that biohydrogenation had been inhibited to a certain point, possibly due to the

build-up of C18:1, *trans*-11 itself. For the most part, there was a suggestion of increased lipolysis with higher concentrations of A1 phospholipase, as 150 µM returned the most significant results.

An accumulation of the intermediate C18:1, *trans*-11 is encouraging as in the bovine mammary gland the Δ9-desaturase enzyme is able to endogenously synthesise CLA using C18:1, *trans*-11 (Griinari*et al.,* 2000). CLA is a human health beneficial fatty acid, known for its anti-carcinogenic properties its major source is found in food products from ruminants (Bauman and Griinari, 1999). Also of particular note for the phospholipase addition experiment was the fact that lipolysis resulted in continual release of fatty acids from the phospholipids, with levels peaking at 24h which is when the greatest reductions in C18:0 were seen. This is interesting as increasing lipolysis may result in the continual release of PUFA, leading to reduced bacterial adaptation and longer term effects upon biohydrogenation compared to initial studies where either LA or LNA were added.

Implementation of phospholipases into the diet of ruminants would nonetheless firstly require *in vivo* studies to clarify levels required to achieve required changes to lipid metabolism. Once confirmed the ability to pellet the phospholipase as a concentrate based supplement would be assessed. Lastly and most importantly the agricultural sector us under immense economic challenges and addition of additives/supplements has to be economically viable. Addition of 150 µM phospholipase to a tonne of concentrate would cost approximately and additional £400 according to current costs of the enzyme from Sigma-Aldrich (UK). This is a substantial addition, but it may be possible to find more effective phospholipases from the rumen bacteria themselves which may improve the cost effectiveness of employing this strategy. The inhibition of lipolysis is also another potential strategy which could be used, and may well be more cost effective.

In summary, this study demonstrates that high concentrations of PUFA are capable of inhibiting biohydrogenation and achieving these levels through enhancement of lipolysishas the potential to reduce SFA content/increase PUFA content (especially CLA) of ruminant products.

4.1: Areas for future study

Firstly the experiments within this thesis need to be repeated to ensure validity of the data. As indicated, the research surrounding lipolysis in the rumen is lacking, and when considering lipolysis as a point of manipulation it is essential that further research is

undertaken, especially to elucidate the mechanism behind PUFA toxicity to rumen microbes. Characterising lipolytic organisms of the rumen would also contribute to our overall knowledge of the rumen consortium in addition to characterising enzymes with potential applications in industry. Further studies similar to Prive (2010) would aid the discovery of novel enzymes, and would allow studies like Huxley's (2012) and the project documented here to further our understanding of lipolysis.

Research into the rumen microbial population has recently gained new and effective culturing techniques and next generation sequencing. Using techniques such as DGGE (Denaturing Gradient Gel Electrophoresis) alongside next generation sequencing, this could make characterisation of the rumen metagenome a reality in the future. Next generation sequencing (NGS) techniques have revolutionised sequencing over the past 5 years, allowing large amounts of DNA (including metagenomes) to be sequenced rapidly and at a much lower cost compared to Sanger sequencing. NGS allows the application of 'culture independent' methods, which are especially useful with regards to the "difficult to culture" rumen microbiome. Large volumes of in-depth data are produced when using NGS, therefore the next challenge is to create ways of dealing with this large amount of data efficiently. Strictly anaerobic culture techniques and culture independent methods, as demonstrated here, could also provide an insight into the uncultured microorganisms of the bovine rumen.

Addressing the practicalities of administering the A1 phospholipase, the concept of pelleting has been suggested, however during the production of concentrates, high temperatures are often used, whilst A1 is able to withstand temperatures up to 60°C this may not be sufficient (BRENDA, 2014). Another option for administration could be in a powder form; however the similar production issues apply. Cattle are also less likely to take in the correct amount using this method, as powders are easily brushed off food items or detected and refused by the animal.

Therefore inhibition may be a suitable alternative to enhancement of lipolysis. The phospholipase A2 has been widely researched, this is a phospholipase that cleaves the second carbon group of glycerol and is found in mammalian tissues as well as insect and snake venom (Balsinde*et al.*, 1999). Lipocortins (or Annexins) have shown great potential in A2 inhibition, lipocortins can be induced naturally by the application of glucocorticoids as well experimentally added. However, lipocortins have not been tested for specificity to A2 alone, and could interact with other mammalian phospholipases; nonetheless perhaps such studies could provide a basis for research into inhibition (Davidson *et al.*, 1999).

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